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Our Proposal aims to investigate the function of VEGF in breast cancer cell growth and signaling. Our data suggest that breast cancer cells secrete various levels of VEGF and express in addition to the Flt-1 and Flk-1/KDR receptors, a novel VEGF receptor type which needs to be identified and characterized. These data lead us to hypothesize that VEGF, secreted by breast cancer cells, is a multi-functional protein which acts in an autocrine fashion and can simultaneously activate specific VEGF receptor signaling pathways in breast cancer cells, thereby regulating breast cancer cell growth, tumor angiogenesis and subsequently tumor growth. In order to test this hypothesis, we propose to focus on two basic aims: (a) To identify and characterize the VEGF receptors expressed in breast cancer cell, and to analyze their expression in primary breast tissues. We will elucidate the signaling events upon VEGF stimulation in breast cancer cells and identify which activated signaling molecules are essential for the VEGF-mediated effects on breast cancer cell growth; and (b) To study the effects of VEGF and VEGF receptor expression on the regulation of tumor angiogenesis and signal transduction pathways in breast cancer cells.

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Title: Studies of Vascular Endothelial Growth Factor (VEGF) Signaling in Breast Cancer Cells

P.I.: Hava Avraham, Ph.D.

INTRODUCTION

The overall goal of our proposal is to investigate the function of VEGF in breast cancer cell growth and signaling. The first step in VEGF action is binding to its high affinity tyrosine kinase receptors Flt-1 and Flk-1/KDR, found primarily in endothelial cells. Our data suggest that breast cancer cells secrete various levels of VEGF and express in addition to the Flt-1 and Flk-1/KDR receptors, a novel VEGF receptor type which needs to be identified and characterized. These data lead us to hypothesize that VEGF, secreted by breast cancer cells, is a multi-functional protein which acts in an autocrine fashion and can simultaneously activate specific VEGF receptor signaling pathways in breast cancer cells, thereby regulating breast cancer cell growth, tumor angiogenesis and subsequently tumor growth. In order to test this hypothesis, we propose to focus on two basic aims: (a) To identify and characterize the VEGF receptors expressed in breast cancer cell, and to analyze their expression in primary breast tissues. We will elucidate the signaling events upon VEGF stimulation in breast cancer cells and identify which activated signaling molecules are essential for the VEGF-mediated effects on breast cancer cell growth; and (b) To study the effects of VEGF and VEGF receptor expression on the regulation of tumor angiogenesis and signal transduction pathways in breast cancer cells. Specifically, we will investigate effects of conditional ectopic expression of VEGF and VEGF receptors in MCF-7 breast cancer cells and in MCF-10A normal mammary epithelial cells and will analyze the effects of their overexpression on the regulation of tumor angiogenesis and signal transduction pathways in breast cancer cells.

RESULTS

In our recent study, we showed that breast tumor cells are capable of responding to VEGF. We show that VEGF stimulation of T-47D breast cancer cells leads to changes in cellular signaling and invasion. VEGF increases the cellular invasion of T-47D breast cancer cells on Matrigel/fibronectin-coated transwell membranes by a factor of two. Northern analysis for the expression of the known VEGF receptors shows the presence of moderate levels of Flt-1 and low levels of Flk-1/KDR mRNAs in a variety of breast cancer cell lines. T-47D breast cancer cells bind ^{125}I-labeled VEGF with a Kd of 13 x 10^{-9} M. VEGF induces the activation of the extracellular regulated kinases 1, 2 as well as activation of phosphoatidylinositol 3'-kinase, Akt, and Forkhead receptor L1. These findings in T-47D breast cancer cells strongly suggest an autocrine role for VEGF contributing to the tumorigenic phenotype (Price et al., Cell Growth & Differentiation 12:129-135).

Heparin/HSPGs were shown to enhance and stabilize the binding of VEGF to its receptors in endothelial and breast cancer cells. However, none of the previous studies have shown whether the enhanced binding of VEGF to its receptors is associated with functional responses in these cells. Only cells passaged on a membrane coat containing fibronectin responded to VEGF by increased migration. Fibronectin and heparin bind to the cell surface through receptors and high affinity sites, respectively. VEGF, VEGF-receptors and fibronectin possess a highly basic region that confers strong affinity for heparin. Furthermore, heparin and HSPGs enhance the binding of VEGF to its receptors and are necessary for the biological actions of b-FGF. Therefore, we hypothesize that T-47D cell responsiveness to VEGF might require the involvement of extracellular matrix components.. We furthermore, examined the effects of ECM components, fibronectin and heparin, on the biological responsiveness of T-47D breast cancer cells to VEGF.

Briefly we analyzed the effects of VEGF in breast cancer cells and the role of ECM components in VEGF induced signaling. Cells grown on plastic were compared to those grown on fibronectin or to those grown on plastic in the presence of heparin, and analyzed for intracellular signaling, proliferation and migration in response to $VEGF_{165}$. c-*fos*, and indicator of cells entering the cell cycle, was examined in addition to mitogenic response and cell invasiveness. Both heparin and fibronectin enhanced the binding of VEGF to T-47D cells. In the presence of VEGF, [^{3}H]thymidine incorporation, c-*fos* induction and the number of migrating cells were significantly higher (~2-fold) in cells grown on fibronectin or in cells grown on plastic in the presence of heparin when compared to those grown on plastic only. Likewise, tyrosine phosphorylation, MAPK activity and P13-kinase activity were all several-fold higher in cells seeded on fibronectin or in the presence of heparin as compared to cells exposed to VEGF alone. VEGF-dependent c-*fos* induction was found to be regulated through a MAPK-dependent, but P13-kinase-independent, pathway. In contrast, the migration of T-47D cells in response to VEGF, in the presence of ECM, was regulated through P13-kinase. Thus, VEGF requires ECM components to induce a mitogenic response and cell migration in T-47D breast cancer cells (see manuscript by Miralem et al., in Press, 2001; Oncogene)

KEY RESEARCH ACCOMPLISHMENTS:

The Effects of VEGF on Signaling, migration, and survival has been analyzed in breast cancer cells. In addition, we determined the role of the extracellular matrix (ECM) in mediating VEGF's function in breast cancer cells.

REPORTABLE OUTCOMES

We have published two papers. See attached both papers.

1) Price DJ, Miralem T, Jiang S, Steinberg R, Avraham H. Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. Cell Growth & Differentiation 12:129-135, 2001.
2) Miralem T, Steinberg R, Price P, Avraham H. VEGF requires extracellular matrix components to induce mitogenic effects and migratory response in breast cancer cells. Oncogene In Press, 2001.

CONCLUSIONS

1) VEGF stimulation of breast cancer cells resulted in increase in cellular invasion and played a role in signaling in breast cancer cells.
2) VEGF required the extracellular matrix components to induce mitogenic effects and migratory response in breast cancer cells.

FUTURE PLAN

We aim to study the effects of VEGF and VEGF expression on the regulation of tumor angiogenesis and signal transduction pathways in breast cancer cells.

Role of Vascular Endothelial Growth Factor in the Stimulation of Cellular Invasion and Signaling of Breast Cancer Cells[1]

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Division of Experimental Medicine, Beth Israel-Deaconess Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts 02115

Abstract

The expression of vascular endothelial growth factor (VEGF) by breast tumors has been previously correlated with a poor prognosis in the pathogenesis of breast cancer. Furthermore, VEGF secretion is a prerequisite for tumor development. Although most of the effects of VEGF have been shown to be attributable to the stimulation of endothelial cells, we present evidence here that breast tumor cells are capable of responding to VEGF. We show that VEGF stimulation of T-47D breast cancer cells leads to changes in cellular signaling and invasion. VEGF increases the cellular invasion of T-47D breast cancer cells on Matrigel/fibronectin-coated transwell membranes by a factor of two. Northern analysis for the expression of the known VEGF receptors shows the presence of moderate levels of Flt-1 and low levels of Flk-1/KDR mRNAs in a variety of breast cancer cell lines. T-47D breast cancer cells bind ^{125}I-labeled VEGF with a Kd of 13×10^{-9} M. VEGF induces the activation of the extracellular regulated kinases 1,2 as well as activation of phosphatidylinositol 3′-kinase, Akt, and Forkhead receptor L1. These findings in T-47D breast cancer cells strongly suggest an autocrine role for VEGF contributing to the tumorigenic phenotype.

Introduction

VEGF[3] is widely recognized to be significant as a stimulator of tumor angiogenesis (1–5). A variety of studies have shown the importance of VEGF as a prognostic indicator of the severity of breast cancer (6–10). VEGF occurs in a number of isoforms, including polypeptides of 121, 145, 165, 189, and 206 amino acids, which are produced by the alternate splicing of a single gene containing eight exons (11–14). Although VEGF-121 and VEGF-165 are the isoforms most commonly secreted by tumor cells, it is the VEGF-165 isoform that acts most strongly on endothelial cells, leading to the formation of new capillaries (15, 16). This effect of VEGF-165 on endothelial cells has been shown to be through defined cytoplasmic receptors (Flt-1, Flk-1/KDR, and Neuropilin-1; Refs. 17–22). Among its other effects, the stimulation of endothelial cells by VEGF-165 is known to lead to cell proliferation and migration (20, 23). These functions are likely to be important in the formation of neovasculature during tumor formation. In support of this, murine embryonic fibroblasts with targeted deletion of VEGF were significantly less tumorigenic in an *in vivo* model, and this was shown to be related to decreased vascular density and decreased vascular permeability (1).

VEGF has been shown to be present in breast tumors at levels that are, on average, 7-fold higher than in normal adjacent tissue (24). Expression of the VEGF receptor, Flt-1, was not increased in these tumors. Other investigators have found selective expression of VEGF and Flk-1/KDR in breast carcinomas (25). Immunocytochemistry showed that Flk-1/KDR was primarily present in the endothelium and epithelium of the mammary ducts. A number of studies have shown that VEGF secretion by the tumor cells is a prerequisite of tumor development. It was shown recently by Yoshiji *et al.* (26) that VEGF was required for the initial stages of breast cancer tumorigenesis, and that this initial effect was related to the development of neovascular stroma. Other studies have shown that the inhibition of vascular angiogenesis by such agents as angiostatin and endostatin resulted in reduced tumorigenesis and even regression of established tumors (27–30).

Although the significance of VEGF in the development of tumor vasculature is well documented, there is also a great amount of information to suggest an autocrine effect of VEGF on the tumor cells. There have been reports of VEGF signaling in melanoma cells (31, 32) and in prostate carcinoma cells (33). Both VEGF and Flt-1 have been shown to be expressed in angiosarcoma cells by immunohistochemistry and *in situ* hybridization (34). In another study, De Jong *et al.* (35) have used immunohistochemistry to measure VEGF and VEGF receptors in breast cancer. They also investigated EGF, PDGFα and -β, TGFβ, and their respective receptors. By carrying out double staining for the receptor/ligand combinations, they were able to distinguish possible autocrine and paracrine mechanisms for VEGF acting on the cells of the tumor. These investigators concluded that in 22–24% of cases, VEGF could act in an autocrine manner, whereas in 38–40% of the cases, it would be able to act in a paracrine

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[1] This paper is supported by NIH Grants CA 76226 and R21CA87290, DAMD 17-98-1-8032, and DAMD 17-99-1-9078, by Experienced Breast Cancer Research Grant 34080057089, by the Milheim Foundation, by the Massachusetts Department of Public Health (to H. A.), and by DAMD 17-001-0152 (to T. M.). This work was done during the tenure of an established investigatorship from the American Heart Association (H. A.).
[2] To whom requests for reprints should be addressed, at Division of Experimental Medicine, Beth Israel-Deaconess Medical Center, Harvard Institutes of Medicine, 4 Blackfan Circle, Boston, MA 02115. Phone: (617) 667-0073; Fax: (617) 975-6373; E-mail: havraham@caregroup.harvard.edu.
[3] The abbreviations used are: VEGF, vascular endothelial growth factor; HRG, heregulin; MTR, Matrigel; PI 3-kinase, phosphatidylinositol 3′-kinase; ERK, extracellular regulated kinase; FKH, Forkhead; FKHRL1, Forkhead receptor L1; HUVEC, human vascular endothelial cell; MAP, mitogen-activated protein; GSK-3, glycogen synthase kinase-3.

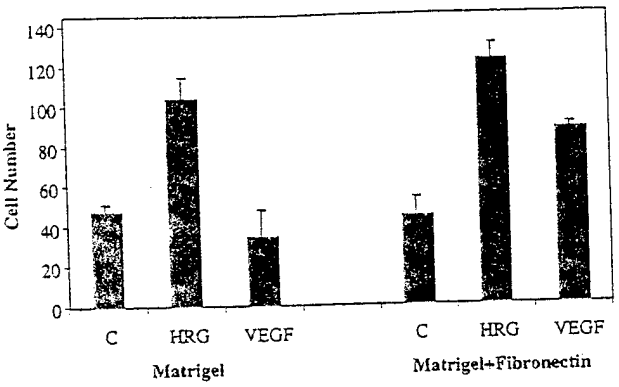

Fig. 1. Invasion of T-47D cells in response to VEGF or heregulin. Comparisons are to medium alone (*C*). Migrations were conducted on membranes coated with Matrigel alone (*left*) or Matrigel plus fibronectin (*right*).

manner. In the studies presented here, we show that VEGF-165 is able to stimulate the invasion of T-47D breast cancer cells into Matrigel. However, there was no effect of VEGF-165 on T-47D cell proliferation. We also show the presence of Flk-1/KDR and Flt-1 mRNAs in a number of breast cancer cell lines. Stimulation of T-47D cells with VEGF-165 led to tyrosine phosphorylation of multiple proteins in crude extracts, activation of ERK1,2 and also activation of the PI 3-kinase signaling pathway. Because T-47D cells are known to secrete VEGF (26), this effect on T-47D cells suggests a possible autocrine component for VEGF, leading to increased tumorigenesis.

Results

VEGF-165 Stimulation Leads to Increased Invasion of T-47D Cells. It was recently reported that VEGF[4] modulates the chemotaxis and migration of endothelial cells (20). In addition, cellular invasion of MCF7 breast cancer cells in response to heregulin has been shown to be mediated through a PI 3-kinase-dependent pathway (36). Therefore, we asked whether VEGF signaling in T-47D breast cancer cells might also affect the invasion of these cells. As is shown in Fig. 1 (*left panel*), initial experiments on Matrigel alone showed no invasion in response to VEGF-165, whereas a >2-fold increase in invasion in response to heregulin was observed. However, when fibronectin was added to the Matrigel coating on the transwell membrane, VEGF-165 caused an ~2-fold increase in invasion that was ~72% of the invasion observed in response to heregulin under these conditions (Fig. 1, *right panel*). These results indicate that VEGF-165 induced the invasion of breast cancer cells in the presence of fibronectin.

Breast Cancer Cell Lines Express Primarily Flt-1 mRNA. We tested for the possible expression of VEGF receptor mRNAs in human breast tumor and normal breast cell lines. Using Northern blotting, we analyzed the expression of Flt-1, Flk-1/KDR, and Neuropilin-1 mRNAs in a number of

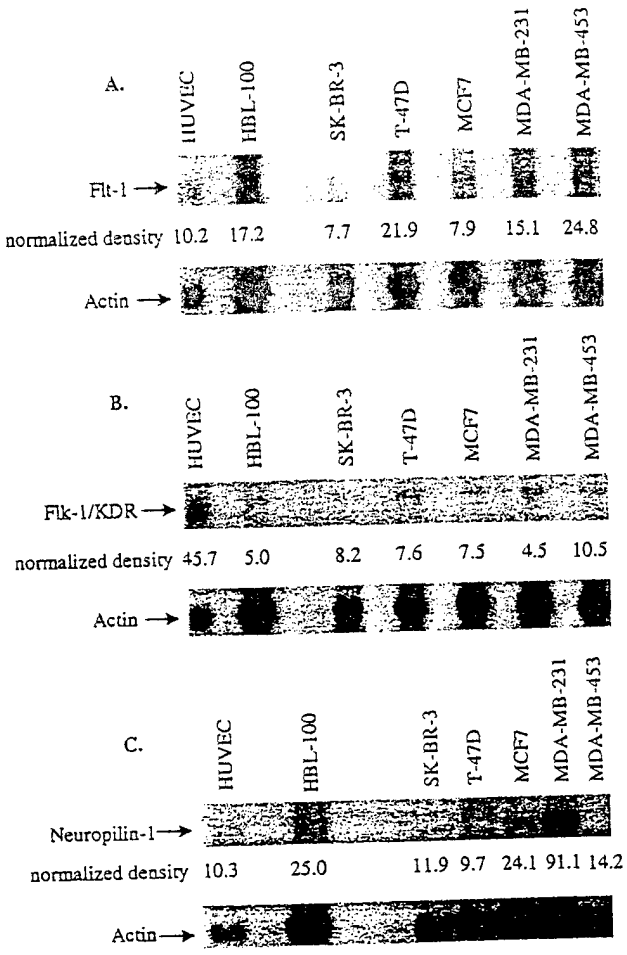

Fig. 2. Northern blotting for (*A*) Flt-1, (*B*) Flt-1/KDR, and (*C*) Neuropilin-1 mRNAs. Cell lines tested are indicated above. Control actin mRNA probe is shown in each *panel*. Densitometry of the Flt-1, Flk-1/KDR, and Neuropilin-1 bands was normalized to the corresponding actin signals.

cell lines. These included SK-BR-3, T-47D, MCF7, MDA-MB-231, and MDA-MB-453 breast cancer lines as well as an HBL-100 nonmalignant breast line and HUVECs. As shown in Fig. 2*A*, all of the breast cancer lines except SK-BR-3 and MCF7 expressed a moderate level of Flt-1. HUVECs expressed a comparatively low level of this mRNA. HUVECs expressed a comparatively high level of Flk-1/KDR, whereas all of the breast cancer cells expressed low levels of this mRNA (Fig. 2*B*). MDA-MB-231 cells expressed a high level of Neuropilin-1, and MCF7 cells expressed a lower level of this mRNA (Fig. 2*C*). Other breast cancer lines failed to express Neuropilin-1 mRNA. These findings indicate that there is variable expression of VEGF receptors (Flt-1, Flk-1/KDR, and Neuropilin-1) in breast cancer. Although T-47D cells and three other breast lines expressed primarily Flt-1, only one of the cell lines (MDA-MB-231) expressed a high level of Neuropilin-1. Flk-1/KDR expression was uniformly low in all of the breast cell lines studied.

VEGF-165 Binds to T-47D Cells with a Lower Affinity Compared with the Known VEGF Receptors. To characterize the possible cellular receptors for VEGF on breast

[4] Notation: Unless otherwise stated, all notation of VEGF refers to the VEGF-165 isoform.

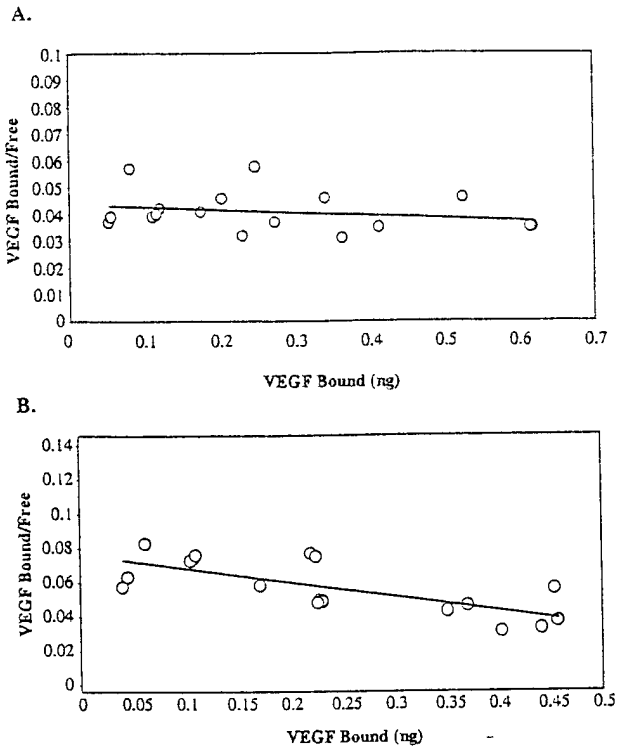

Fig. 3. Scatchard analysis of binding of ^{125}I-VEGF to MDA-MB-231 cells (*A*) and T-47D cells (*B*). Lines indicate a least square fit of the data points.

cancer cells, we determined the binding of ^{125}I-labeled VEGF to either T-47D cells or to MDA-MB-231 cells as a control. From Scatchard analysis of this data (Fig. 3), we calculated the binding of VEGF to T-47D cells as having a Kd of $\sim 13 \times 10^{-9}$ M and $\sim 0.63 \times 10^5$ binding sites/cell. Binding of VEGF to MDA-MB-231 cells showed a Kd of $\sim 17.4 \times 10^{-10}$ M and $\sim 1.53 \times 10^5$ binding sites/cell. Thus, MDA-MB-231 cells had a VEGF binding that was similar to that determined previously by Soker *et al.* (Ref. 21; Kd $\sim 2.8 \times 10^{-10}$ M; 0.95–1.1 $\times 10^5$ binding sites/cell) reflecting the binding primarily to Neuropilin-1. To confirm our binding data obtained for detached cells, we repeated the experiments following more closely the method of Soker *et al.* (21), who determined binding to cells on tissue culture wells. Using this method, we obtained Kd values for VEGF binding to MDA-MB-231 and T-47D cells that were similar (within a factor of 2–3) to the values obtained by our method with detached cells (data not shown). Waltenberger *et al.* (20) have characterized VEGF binding to Flt-1 to have a Kd of 1.6×10^{-11} M and VEGF binding to Flk-1/KDR to have a Kd of 7.6×10^{-10} M. Our experiments with T-47D cells, on the other hand, showed a binding that was lower in affinity as compared with all of the known VEGF receptors.

VEGF-165 Stimulates the Tyrosine Phosphorylation of a Number of Proteins in T-47D Breast Cancer Cells. We next asked whether VEGF might have an effect on the signaling of receptor tyrosine kinases to intracellular components in T-47D cells. Thus, we stimulated these cells with VEGF-165 and measured the changes in total tyrosine phosphorylation. When serum-starved T-47D cells were treated with 100 ng/ml VEGF-165, we found increased tyrosine phosphorylation of a number of proteins as shown by anti-phosphotyrosine Western blotting of total cell extracts (Fig. 4). These included proteins of molecular weight M_r 60,000, 75,000, 122,000, and 200,000.

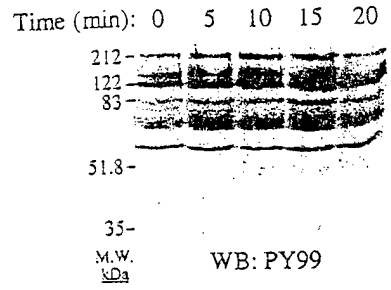

Fig. 4. Stimulation of T-47D cells with VEGF and western immunoblotting of total cell extracts with an anti-phosphotyrosine antibody.

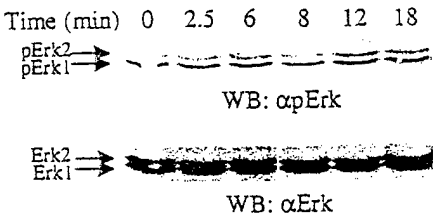

Fig. 5. Stimulation of T-47D cells with VEGF and Western immunoblotting of total cell extracts with anti-phospho-ERK antibody (*upper panel*). *Lower panel* indicates immunoblotting with an antibody to total ERK 1 protein.

VEGF-165 Stimulates the MAP Kinases ERK 1,2 in T-47D Breast Cancer Cells. To analyze whether VEGF might modulate MAP kinase activity in the breast cancer cells, extracts from T-47D cells activated with VEGF were resolved on SDS-PAGE, and transfers were probed to detect activation of ERK 1,2 (Fig. 5). We observed a slight increase in the phosphorylation of ERK 1,2 that was attributable to VEGF at 15–20 min. Blotting for total ERK 1,2 showed that the differences seen were not attributable to differences in protein loading.

VEGF-165 Treatment Leads to Stimulation of PI 3-kinase and Related Pathways in T-47D Breast Cancer Cells. Inasmuch as PI 3-kinase has been shown to be induced by VEGF in endothelial cells (37), we next determined whether VEGF also might activate this pathway in breast cancer cells. PI 3-kinase activity was measured by an *in vitro* kinase assay of extracts from the VEGF-treated cells (Fig. 6*A*, *left panel*). There was a clear stimulation of phosphatidylinositol phosphorylation by VEGF at 5–20 min (see *arrow*). There was no phosphorylation seen in the normal serum control precipitate. As a control, we stimulated T-47D cells with heregulin and measured the PI 3-kinase activity (Fig. 6*A*, *right panel*). This showed the position of phosphatidyl inositol 3-phosphate in the chromatogram.

Because Akt is known to be a down-stream target of PI 3-kinase (38), we then measured Akt activation in VEGF-

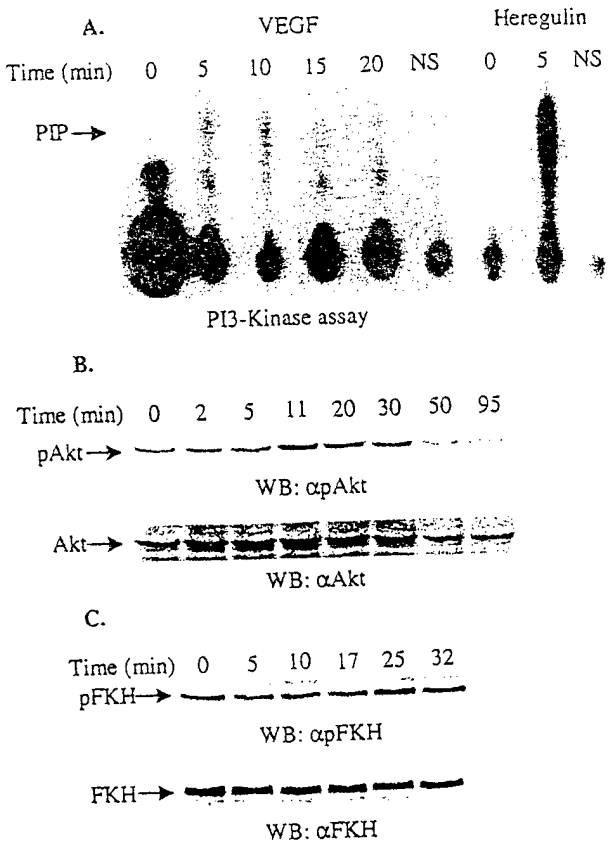

Fig. 6. (*A*) Stimulation of T-47D cells with VEGF and assay for PI 3-kinase (*left panel*). *Right panel* indicates the PI 3-kinase assay of T-47D cells stimulated with heregulin. NS indicates normal serum control precipitate. (*B*) Stimulation of T-47D cells with VEGF and immunoblotting of extracts with anti-phospho-Ser253 Akt. (*C*) Stimulation of T-47D cells with VEGF and immunoblotting of extracts with anti-phospho-FKH antibodies. *Below* each is shown the corresponding blotting of the non-phosphorylated protein.

treated T-47D cells by Western blotting using a phospho-Akt antibody (Fig. 6*B*). VEGF produced a small but detectable Akt phosphorylation signal that was first seen at 25 min, decreased at 50 min, and reached basal levels by 95 min.

We next tested for possible substrates of Akt, including GSK-3, p70 S6 kinase, and FKHRL1. Whereas stimulation of GSK-3 was seen after heregulin treatment, no stimulation of GSK-3 was detectable after VEGF treatment (data not shown). No change was seen in the phosphorylation of p70 S6K at Thr-421 or Ser-424 after either heregulin or VEGF treatment (data not shown). We then tested to see if there was a change in the phosphorylation of FKHRL1, a Forkhead family member known to be involved in the transcription of apoptosis-related proteins (39–41). We saw increases in the phosphorylation of FKHRL1 at Ser-253 in extracts from cells stimulated by VEGF (Fig. 6*C*). A similar increase in phosphorylation was also seen at Thr-32 of FKHRL1 (data not shown). These changes in FKHRL1 phosphorylation appeared to follow the changes in Akt phosphorylation, indicating that FKHRL1 was the substrate for Akt upon VEGF stimulation of breast cancer cells.

Taken together, the results indicate that VEGF induces the activation of ERK1,2 and PI 3-kinase signaling pathways in breast cancer cells, leading to an increased invasiveness of the cells.

Discussion

In this study, we have presented results showing the VEGF-induced invasion and signaling in T-47D breast cancer cells. These studies demonstrate the importance of VEGF in stimulating effects on breast tumor cells in contrast to its effects on endothelial cells. Our studies with mRNA expression show that all breast tumor lines examined, except SK-BR-3, expressed moderate levels of Flt-1 and lower levels of Flk-1/KDR. Thus, we postulate that breast cancer cell lines are representative of human breast tumors in terms of their expressing VEGF receptors. Our studies with T-47D breast cancer cells support the conclusion that these cells are capable of responding to VEGF in terms of changes in intracellular signaling and cellular invasion. Data from our laboratory[5] and from other investigators (26), have shown that T-47D cells secrete VEGF at the levels that are required for this stimulation. Thus, we postulate that, in many breast cancers, the elements are present for stimulation of an autocrine mechanism leading to increased cell invasion.

We tested to see if VEGF receptors other than Flt-1 and Flk-1/KDR might account for the signaling in the breast cancer cells. By Northern blotting, the VEGF receptor Neuropilin-1 was seen to be expressed in two of the breast cancer cell lines, MDA-MB-231 and MCF7 cells. MDA-MB-231 cells contain the highest level of Neuropilin-1, but VEGF fails to stimulate tyrosine phosphorylation or ERK 1,2 activation.[6] Thus, it is unlikely that Neuropilin-1 is involved in the effects that we have seen in T-47D cells. Our Northern blotting of breast cancer cell lines showing the presence of Flt-1 and Flk-1/KDR are consistent with the finding of Speirs and Atkin (42), who found that these receptors were present in human breast cancer tumor epithelial cells. It was similarly shown by De Jong *et al.* (35) that in nearly 50% of the breast tumors, there was significant expression of Flt-1 and Flk-1/KDR in the tumor epithelial cells, correlating with the expression of VEGF by these cells. These investigators postulated that VEGF secreted by these epithelial cells could have both autocrine and paracrine roles. The paracrine mechanism for this action is likely to be through the stimulation of endothelial cells, leading to a development of the neovasculature (17–19, 22). However, the mechanism for the autocrine action of VEGF on the epithelial cells of the tumors has not been characterized. On the basis of the results presented here, we propose that VEGF acts in an autocrine manner by stimulating signaling, leading to cellular invasion in breast cancer epithelial cells. The cellular signaling in T-47D cells stimulated by VEGF leads to the stimulation of ERK1,2 and PI 3-kinase pathways. Stimulation of the PI 3-kinase pathway in particular is often related to cellular invasion. As mentioned above, MCF7 breast cancer cells are known to migrate in response to heregulin through a PI 3-kinase-mediated process (36). We have observed the invasion of T-47D cells in

[5] D. J. Price, H. Kawai, and H. Avraham, unpublished results.
[6] D. Price, unpublished data.

response to VEGF only when fibronectin is present on the transwell membrane. This is an indication that both the growth factor, VEGF, and the extracellular matrix component, fibronectin, are important in potentiating the invasion of the tumor cells. Fibronectin is known to contain binding domains that interact with cell surface heparan sulfate proteoglycans to promote focal adhesions and stress fiber formation (43). Heparan sulfate is known to potentiate the binding of VEGF to its receptors (31). It may be that fibronectin, in conjunction with heparan sulfate proteoglycans, also leads to an increased interaction of VEGF with its receptor. Thus, fibronectin, in cooperation with VEGF, appears to provide the signaling that is required for cellular invasion, whereas VEGF alone is unable to stimulate this process.

To date, we have no indication that other cellular functions might be stimulated in these cells, leading to increased tumorigenesis. There appeared to be little effect on cell survival or proliferation upon VEGF treatment of the T-47D cells (data not shown). Although phosphorylation of the Forkhead transcription factor is often connected with effects on the Fas ligand leading to cell survival (41), there may be other functions of this pathway. Another important question raised by these results is whether or not the endogenously secreted VEGF is sufficient to stimulate the effects that we have observed. As noted above, we and other investigators have shown that VEGF is secreted by the T-47D cells. An argument that could be made about the significance of the effect of VEGF on tumor cells is that because the tumor is secreting VEGF, there may be a higher local concentration of VEGF relative to other growth factors. Thus, *in vivo*, the effect of VEGF on the invasion of these cells may be much greater as compared with the effects of other growth factors that are present at subthreshold concentrations.

In summary, VEGF stimulated the increased invasion of T-47D cells through Matrigel/fibronectin-coated membranes. Northern analysis showed the expression of primarily the VEGF receptor, Flt-1, in a variety of breast cancer cell lines. However, binding of ^{125}I-labeled VEGF to T-47D cells indicated an affinity that was lower than that expected for the known VEGF receptors, suggesting the possibility of an as-yet unidentified VEGF receptor in these cells. VEGF stimulated signaling in T-47D breast cancer cells through the PI 3-kinase/Akt pathway and also through the ERK 1,2 pathway. This observation may indicate an effect of VEGF on tumorigenicity independent of its effects on the vasculature. Future studies will be aimed at characterizing the *in vivo* significance of VEGF signaling in the tumor cells as compared with its signaling in endothelial cells.

Materials and Methods

Materials. Antibodies used in immunological analysis were as follows: anti-phosphotyrosine antibody (PY99), phospho-ERK (E-4) antibody, anti-ERK 1 (K-23), anti-Flk-1 (N-931), and horseradish peroxidase-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt, and anti-phospho-Ser-473 Akt antibodies were from New England Biolabs (Beverly, MA). Anti-Flt-1, anti-phospho-Ser-253 FKH, and anti-phospho-Thr-32 FKH antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). LY294002 was from Sigma Chemical Co. (St. Louis, MO). VEGF-165 and heregulin were a generous gift of Genentech (San Francisco, CA). γ^{32}P-ATP and α^{32}P-CTP were from New England Nuclear (Boston, MA). All other chemicals were from Fisher Scientific (Norcross, GA), unless otherwise noted.

Cell Culture. T-47D cells were an estrogen receptor-positive clone provided by Iafa Keydar, Tel Aviv University (Ramat Aviv, Israel). These cells were cultured in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) supplemented with 7 μg/ml insulin, 10% fetal bovine serum (Life Technologies, Inc.), and penicillin/streptomycin. MCF7 cells (American Type Culture Collection, Rockville, MD) were grown in MEM (Life Technologies, Inc.) supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 μg/ml insulin, 10% fetal bovine serum, and penicillin/streptomycin. MDA-MB-231 and MDA-MB-453 (American Type Culture Collection) were grown in DMEM supplemented with 10% fetal bovine serum, 0.2 mM glutamine, and penicillin/streptomycin. HUVECs were from Clonetics (San Diego, CA) and were cultured in EGM complete medium (Clonetics). HBL-100 (American Type Culture Collection) were cultured in McCoy's 5a medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Iodination of VEGF-165. ^{125}I-labeled VEGF-165 was prepared using IODO-GEN, as described previously (31). The protein was separated from free iodine by heparin Sepharose affinity adsorption (Amersham-Pharmacia Biotech, Piscataway, NJ) and elution with 0.8 M NaCl. Specific activity of the ^{125}I-labeled VEGF-165 was ~100,000 cpm/ng protein.

Binding of VEGF-165 to Cells. For quantification of the binding of ^{125}I-labled VEGF-165 to cells, the cells were detached briefly with trypsin/EDTA, washed in full medium, then suspended in binding buffer [20 mM MOPS (pH 7.4)/2 mM $MgCl_2$/140 mM NaCl, and 0.2% gelatin/2 mg/ml glucose). Cells were then incubated with a range of concentrations of VEGF-165 containing a fixed amount of ^{125}I-labled VEGF-165 in binding buffer at a final concentration of 1×10^5 cells/ml on ice. Aliquots of 0.15 ml were pipetted onto a 0.9 cushion of fetal bovine serum. After centrifugation in a microcentrifuge (5 min; 7.5 × 1000 rpm), tubes were frozen on dry ice. The cell pellet was isolated by clipping the tip of the tube with a canine toenail clipper. Bound (pellet) and free (supernatant) counts were quantified in a Beckman gamma counter. Kd values and the number of binding sites/cell were calculated from Scatchard plots (44) by doing a least square fit of the data using the Microsoft Excel program.

Immunoprecipitations and Western Analysis. After growth factor stimulation, cells were lysed in 20 mM Tris-HCl (pH 7.4)/150 mM NaCl/1% NP-40/0.25% deoxycholate/1 mM Na_3VO_4/1 mM EGTA and a cocktail of protease inhibitors (Complete, EDTA-free; Roche, Indianapolis, IN). Protein was normalized by Bio-Rad protein assay (Bio-Rad, Hercules, CA), and lysates were precipitated overnight with the addition of 1 μg of the specified antibody. The next day, protein G-Sepharose (Pierce, Rockford, IL) was added and the precipitates were washed 3 times with lysis buffer. Precipitates were treated with SDS-sample buffer and run on polyacrylamide gels, followed by transfer to nitrocellulose membranes

(Bio-Rad). Membranes were immunoblotted with primary antibodies as indicated in the figure legends, and with the appropriate horseradish peroxidase-linked secondary antibodies, before chemiluminescent development and exposure to X-ray film.

PI 3-kinase Assay. Assay of PI 3-kinase was carried out after growth factor stimulation of cells and precipitation of lysates by PY99 antibody/Protein G-Sepharose. Precipitates were subjected to an *in vitro* kinase reaction using γ^{32}P-ATP and phosphatidylinositol (Sigma Chemical Co.) as substrates, according to Derman *et al.* (45). ^{32}P-labeled samples were applied to oxalate-coated cellulose/acetate plates and subjected to chromatographic separation (solvent, $CHCl_3$: methanol:H_2O:NH_4OH [60:47:11.3:2]).

Northern Analysis. mRNAs were isolated from cellular extracts by oligo(dT) chromatography using a kit (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. mRNAs were separated on an agarose gel and transferred to a Hybond N membrane (Amersham Pharmacia Biotech). The membrane was hybridized with probes to Flt-1, Flk-1/KDR, and Neuropilin-1 (a generous gift of Dr. Michael Klagsbrun, Children's Hospital, Boston, MA). Blots were prehybridized for 4 h at 42° in 50% formamide/5× SSC-10× Denhardts/0.3% SDS/100 μg/ml ssDNA/10 μg/ml yeast tRNA. Specific ^{32}P-labeled probe DNA was added, and the incubation was continued for 4 h at 42°. The blots were washed twice in 2× SSC-1% SDS at room temperature, and then in 0.2× SSC-0.1% SDS at 42° followed by 0.2× SSC-0.1% SDS at 60°. After washing, the blots were then exposed to X-ray film. Blots were also stripped and reprobed for actin mRNA as a control.

Invasion Assay. Transwell membranes (8-μm pore size, 6.5-mm diameter; Corning Costar Corporation, Cambridge, MA) were coated with Matrigel (2.5 mg/ml) or Matrigel plus fibronectin (2.5 mg/ml), and dry coatings were reconstituted in DMEM for 1–2 h before cell passage. Cells were trypsinized, centrifuged, and resuspended at ~10^7/ml in DMEM containing 0.2% BSA. Cells were seeded onto the upper wells of precoated transwells in the same medium alone [control or in medium supplemented with HRG (20 nM)] or VEGF-165 (100 ng/ml). Lower wells of the transwells contained 600 μl of DMEM and 0.2% BSA. After 24 h, membranes were swabbed with a Q-tip, fixed with methanol, and stained with crystal violet before counting under phase-contrast microscopy.

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Dr. Hava Avraham
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Re: Manuscript 10242

Dear Dr. Avraham:

Regarding your manuscript entitled "$VEGF_{165}$ requires extracellular matrix components to induce mitogenic effects and migratory response in breast cancer cells" by Miralem *et al.*, I am pleased to inform you that the you have adequately addressed the reviewers' concerns. I have, therefore, forwarded the manuscript to the Editor-in-Chief, Dr. John Jenkins, with my recommendation for publication in *Oncogene*.

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VEGF Requires Extracellular Matrix Components to Induce Mitogenic Effects and Migratory Response in Breast Cancer Cells

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The abbreviations used are: VEGF, vascular endothelial growth factor; HRG, heregulin; MTR, matrigel; ECM, extracellular matrix; GAG, glycosaminoglycan; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HSPG, heparan sulfate proteoglycan; FN, fibronectin, MEK, mitogen-activated kinase-kinase; AKT, Akt kinase; a-, b-FGF, acidic-, basic-fibroblast growth factor.

Notation: Unless otherwise stated, all notation of VEGF refers to the $VEGF_{165}$ isoform.

Running title: Effect of ECM and VEGF in breast cancer

Key Words: VEGF/breast cancer/cell signaling/mitogenic response/cell migration

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ABSTRACT

The expression of VEGF and the relapse-free survival rate of breast cancer patients are inversely related. While VEGF induces the proliferation and migration of vascular endothelial cells, its function in breast cancer cells is not well elucidated. Some breast cancer cells, including T47D cells, secrete VEGF and express its receptors. Since the extracellular matrix (ECM) plays an important role in the development of breast cancer *in vivo*, we sought to determine the effects of VEGF in breast cancer cells and the role of ECM components in VEGF-induced signaling. Cells grown on plastic were compared to those grown on fibronectin or to those grown on plastic in the presence of heparin, and analyzed for intracellular signaling, proliferation and migration in response to $VEGF_{165}$. c-*fos*, an indicator of cells entering the cell cycle, was examined in addition to mitogenic response and cell invasiveness. Both heparin and fibronectin enhanced the binding of VEGF to T47D cells. In the presence of VEGF, [^{3}H]thymidine incorporation, c-*fos* induction and the number of migrating cells were significantly higher (~2-fold) in cells grown on fibronectin or in cells grown on plastic in the presence of heparin when compared to those grown on plastic only. Likewise, tyrosine phosphorylation, MAPK activity and PI3-kinase activity were all several-fold higher in cells seeded on fibronectin or in the presence of heparin as compared to cells exposed to VEGF alone. VEGF-dependent c-*fos* induction was found to be regulated through a MAPK-dependent, but PI3-kinase-independent, pathway. In contrast, the migration of T47D cells in response to VEGF, in the presence of ECM, was regulated through PI3-kinase. Thus, VEGF requires ECM components to induce a mitogenic response and cell migration in T47D breast cancer cells.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is angiogenic in vitro (1) and in vivo (2) by inducing the proliferation (3) and migration (4) of vascular endothelial cells. VEGF is produced in numerous cell types such as tumor cells, smooth muscle cells, mesangial cells, macrophages, and osteoblasts (5). It is a homodimer (6) that occurs in five isoforms specifically: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, as a result of alternative splicing from a single gene (7). Of these, $VEGF_{121}$ and $VEGF_{165}$ are the most abundant and potent isoforms (8). While $VEGF_{121}$ lacks an ECM-binding domain and is secreted only in medium, $VEGF_{165}$ is found to be both cell-associated and secreted in medium (9).

The biological effects of VEGF are elicited through the Flt-1 and KDR high-specificity receptors (10). These receptors are expressed predominantly in endothelial cells (11) and in several other cell types (12), including breast cancer cells (13,14). Both receptors have an extracellular domain containing seven immunoglobulin-like loops and an intracellular domain characterized by split tyrosine kinase motifs (10). Upon activation, these receptors dimerize and autophosphorylate at their tyrosine residues in the intracellular C-terminus (15). The phosphorylated tyrosines act as docking sites for adaptor-signaling molecules and non-receptor kinases, thereby generating signaling cascades. Thus, VEGF activates the MAPK pathway as a key component in generating signals for growth and transformation (16) and also the PI3K/Akt pathway (17) as a regulator of cell survival (18) and migration (4).

VEGF belongs to the fibroblast growth factor (FGF) group of growth factors characterized by their binding affinity for heparin (19,20). Such binding affinity was shown to have a biological effect, enabling a productive interaction between the growth factor and its cell-

surface receptors (21,22). Heparinase treatment of endothelial cells inhibited endothelial cell proliferation and *in vivo* neovascularization (23), emphasizing the role of heparin-like molecules in angiogenesis. The presence of heparin-like molecules is essential for the binding of b-FGF to its receptors (22,24), thereby protecting b-FGF from heat and acidic inactivation (25). The interaction of VEGF with heparin is more modest as compared to that of b-FGF (21,22), and is comparable to the modest affinity shown by PDGF, a member of the same group of growth factors (26). Although having a relatively weak interaction with VEGF, heparin and heparan sulfate proteoglycans (HSPGs) are able to enhance the interaction of VEGF with its receptors (27). The binding of VEGF to its cell-surface receptors was restored by the addition of heparin after the concentration of heparin-like molecules on the cell surface was reduced by heparinase treatment (27,28). However, the binding of $VEGF_{121}$, a truncated variant that lacks a heparin-binding domain, could not be restored by the addition of heparin-like molecules (29).

Breast cancer cells synthesize VEGF and express VEGF receptors (13,30). Heparin/HSPGs were shown to enhance and stabilize the binding of VEGF to its receptors in endothelial and breast cancer cells (28,30). However, none of the previous studies have shown whether the enhanced binding of VEGF to its receptors is associated with functional responses in these cells. Only cells passaged on a membrane coat containing fibronectin responded to VEGF by increased migration (13). Fibronectin (31) and heparin (32) bind to the cell surface through receptors and high affinity sites, respectively. VEGF (33), VEGF-receptors (34), and fibronectin (35) possess a highly basic region that confers strong affinity for heparin. Furthermore, heparin and HSPGs enhance the binding of VEGF to its receptors (28) and are necessary for the biological actions of b-FGF (22,24). Therefore, we hypothesize that T47D cell responsiveness to VEGF might require the involvement of extracellular matrix components. This study was

undertaken to examine the effects of ECM components, fibronectin and heparin, on the biological responsiveness of T47D breast cancer cells to VEGF.

EXPERIMENTAL PROCEDURES

Materials. Antibodies used for immunological analysis were as follows: anti-phosphotyrosine antibody (4G10) was from Genentech (San Francisco, CA). Phospho-Erk (E-4) antibody, anti-Erk-2 (C-154), anti-Flt-4 antibody, anti-Flk-1 antibody and HRP-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt, and anti-phospho-Ser-473 Akt antibodies were from New England-Biolabs (Beverly, MA). Anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). Genistein, LY294002, heparin, chondroitin sulfate A, and C, dextran sulfate, and phosphatidylinositol were from Sigma Chemical Co. (St. Louis, MO). PD98059 was from Calbiochem-Novobiochem Co. (La Jolla, CA). $VEGF_{165}$ and heregulin were generous gifts from Genentech. $\gamma[^{32}P]ATP$, $\alpha[^{32}P]dCTP$, $[^{3}H]$thymidine and $[^{125}I]Na$ were from New England Nuclear (Boston, MA). All other chemicals were from Fisher Scientific (Norcross, GA), unless otherwise specified.

Cell Culture. T47D cells were obtained from ATCC. These cells were cultured in RPMI-1640 medium (Gibco-BRL), supplemented with 3.5 μg/ml insulin, 10% fetal bovine serum (Gibco-BRL), and penicillin/streptomycin. To coat the plates: human plasma fibronectin (30 μg/ml), collagen type IV (40 μg/ml) and Matrigel (Engelberth-Holm-Swarm tumor basement membrane, 40 μg/ml) (Becton-Dickinson, Bedford, MA) were dissolved in sterile water, 0.05 M HCl or in cold medium, respectively, spread on culture dishes (1.2 ml/10-cm petri dish, 70 μl/well in 24-well plates), and allowed to dry in a sterile environment. Collagen type I solution (2.9 mg/ml) was purchased as Vitrogen-100 (Cohesion, Palo Alto, CA) and was added to the wells of the tissue culture plates (0.25 ml/well in 24-well plates), incubated for at least 60 min at 37°C to gel. Poly-L-lysine (40 μg/ml, Sigma) was dissolved in water spread on culture dishes (70 μl/ml in 24-

well plates) and allowed to dry in a sterile environment. After gelling or drying of the matrix coat, plates were washed twice with PBS and once with RPMI-1640 to remove unbound proteins before the addition of the cell suspension. Quiescence was induced by replacing growth medium on cells at 60-80% confluence with medium containing 0.4% FBS, then followed by 48 h incubation. To study the initiation of signaling, cells were stimulated with either FBS (10%), VEGF (100 ng/ml) alone or in the presence of heparin, chondroitin sulfate, or dextran sulfate (all at a concentration of 1 μg/ml) or with heregulin (20 nM).

Cell attachment. Cell attachment was performed as described (36). Briefly, cells were released from petri dishes by trypsinization, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin (BSA; 1 mg/ml) and then seeded in 12-well plates (5 X 10^6 cells per plate) that were either uncoated or precoated with fibronectin (30 μg/ml). The cells were allowed to attach for 60 min, then unattached cells were removed, and attached cells were released by trypsin/EDTA. The percentage of attached cells was measured by both the direct counting of attached and unattached cells using a Coulter counter or by scintillation counting of radiolabeled cells as described by Grinnell and Feld (37). The percent attachment was calculated as 100X[attached/(attached+unattached)].

Radioiodination. The iodination of $VEGF_{165}$ was carried out using IODO-GEN (Pierce, Rockford, IL) as described previously (30). ^{125}I-VEGF was then purified by heparin affinity chromatography in the presence of gelatin (10 μg/ml). Aliquots of the iodinated VEGF were frozen on dry-ice and stored at –70°C until needed. The specific activity of ^{125}I-VEGF was approximately 5 x 10^4 cpm/ng protein.

Binding and cross-linking. T47D cells were grown in 24-well plates or in 60 mm petri dishes. After reaching confluency, they were transferred to an ice-cold environment and washed twice

with ice-cold PBS. ^{125}I-VEGF (10 ng/ml) was added to F12 medium containing 25 mM HEPES (pH, 7.5) and 0.2% gelatin, and cells were then incubated for 4 h at 4°C. At the end of the incubation, cells were washed three times with ice-cold PBS supplemented with 0.1% BSA, lysed in buffer containing 1% Triton X-100 and 0.1% BSA, and counted in a γ counter for recovered radioactivity. For the cross-linking studies, following incubation for 4 h in 60 mm plates, cells were treated with PBS containing disuccinimidyl suberate (0.15 mM) at room temperature. After 15 min, the reaction was stopped by the addition of 200 µl of quenching buffer (10 mM Tris-HCl, pH 7.5, 200 mM glycine, and 2 mM EDTA). Cells were then washed with ice-cold PBS, scraped in PBS containing 1 mM PMSF and 1 mM EDTA and centrifuged for 30 seconds prior to dissolving pellets in lysis buffer (10 mM Tris-HCl, pH 7.0, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.1 mM EDTA, and 1 mM PMSF). The suspension was centrifuged as before and aliquots from the supernatant were analyzed by SDS-PAGE followed by autoradiography.

Mitogenic response. Mitogenic response of T47D cells was performed as previously described (36). Briefly, cells were passaged at 10^5 cells/well (24-well plate) and grown either on plastic, fibronectin, collagen type I or IV, Matrigel, or poly-L-lysine-coated plates to 60-70% confluence at which time the serum content of the medium was lowered to 0.4% to arrest cell growth. After 48 h, a mitogenic response in quiescent cells was induced with VEGF (100 ng/ml), heregulin (HRG, 20 nM), or fetal bovine serum (FBS, 10%), and measured by [^{3}H]thymidine (6.7 Ci/mM, 2 mCi/ml for 45 min) incorporation at different time points up to 24 h. After being labeled with [^{3}H]thymidine, cells were washed three times with 5% trichloroacetic acid at 0°C, dissolved with 0.1 M NaOH, and radioactivity was measured by using scintillation counter.

RNA isolation and Northern blotting. Total RNA was isolated using an RNA kit from QIAGEN. The electrophoresis and blotting were done as described (38). Briefly, equal amounts of RNA (~ 15 μg) were denatured, separated by electrophoresis on agarose-formaldehyde gels, and transferred to a Hybond-N nylon membrane (Amersham-Pharmacia Biotech). Membranes were hybridized with c-*fos* cDNA that was labeled with $\alpha[^{32}P]$dCTP. Levels of mRNA were normalized to 18S rRNA after probing stripped blots with labeled cDNA to rat 18S rRNA. ^{32}P labeling of probes was carried out with a random primer DNA labeling kit from Boehringer-Mannheim (Indianapolis, IN). The rat c-*fos* cDNA, cloned by T. Curran (11), and the mouse 18S rRNA were obtained from D.M. Templeton (University of Toronto, Ontario).

MAPK activity. Cells were washed twice with ice-cold PBS and scraped into 700 μl of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% (vol/vol) Nonidet P-40, 0.25% (wt/vol) sodium deoxycholate, 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1 μg/ml leupeptin], and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). Cell lysates were sonicated and centrifuged at 100,000 x g for 15 min. After cytosols were assayed for proteins, aliquots of 1 mg protein were precleared by adding 1.0 μg normal rabbit IgG followed by 20 μl of protein G-Agarose, and then centrifuged. The supernatant was incubated with 2 μg of polyclonal rabbit-rat Erk-2 antibody overnight at 4°C, and immunoprecipitates were recovered by incubating with a 50% slurry of protein G-Agarose for a further 4 h. Portions of the immunoprecipitates were subjected to 10% SDS-PAGE according to Laemmli (39), and transferred to polyvinylidene difluoride (PVDF) membranes for Western blotting with anti-Erk-2 antibody as described below. MPAK activity was determined by the ability of the immunoprecipitated enzyme to phosphorylate myelin basic protein (MBP) (40) in an *in vitro* kinase assay. Immunoprecipitates

were mixed with assay buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.5 mM EGTA, 10 mM NaF, 0.5 mM Na_3VO_4, 1 mM dithiothreitol, 0.5 mM mg/ml MBP, 100 mM ATP, and 5 μCi of γ[^{32}P]ATP, and incubated at 30°C for 30 min. The reaction was stopped by the addition of sample buffer for electrophoresis according to Laemmli (39), and the mixture was separated on 15% SDS-PAGE for silver staining and autoradiography.

PI3K activity. Cells were lysed in buffer A containing 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 mM PMSF and 0.1 mM Na_3VO_4, and then cytosolic extracts were subjected to immunoprecipitation with antiphosphotyrosine antibody (PY20). Precipitates were subjected to an *in vitro* kinase assay using γ[^{32}P]ATP and phosphatidylinositol as substrates, according to Derman et al. (41). Briefly, beads were washed and incubated for 10 min at room temperature in kinase buffer containing 0.5 mM ATP, 20 mM $MgCl_2$, 50 mM HEPES, pH 7.0, 0.25 mg/ml phosphatidylinositol and 30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). Lipids were then extracted by $CHCl_3$:CH_3OH (1:1) mixture, separated on oxalate-coated thin-layer chromatography plates (EM Science, Gibbstown, NJ) in developing solution containing $CHCl_3$:CH_3OH:H_2O:NH_4OH [60:47:11,3:2], followed by autoradiography.

Immunoblotting. Cells were lysed in the lysis buffer A, and lysates were subjected to SDS-PAGE according to Laemmli (39). Separated proteins were transferred to PVDF membranes in 25 mM Tris and 192 mM Glycine (pH 8.3) containing 15% methanol, and then blocked with 5% BSA and 5% Carnation milk in 30 mM Tris-HCl (pH 7.4) containing 137 mM NaCl, 2.6 mM KCl and 0.05% Tween 20. Membranes were then probed with either anti-Erk-2 antibody, polyclonal rabbit anti-phospho-Erk antibody, monoclonal mouse anti-phosphotyrosine antibody 4G10, monoclonal mouse anti-actin antibody, polyclonal rabbit anti-Akt, polyclonal rabbit anti-Flt-4 antibody, monoclonal mouse anti-Flk-1 antibody or polyclonal rabbit anti-phospho-Ser-473

Akt antibodies, and immunoreactive bands were detected with the NEN-Biolab (Boston, MA) enhanced chemiluminescence system, followed by autoradiography.

Migration assay. Transwell membranes (Corning Costar Corporation, Cambridge, MA) were coated with matrigel (MTR; 2.5 μg/ml) or MTR plus fibronectin (30 μg/ml), and dry coatings were exposed to DMEM for 1-2 h prior to cell passage. Cells were trypsinized, centrifuged and resuspended at approximately 10^7/ml in DMEM containing 0.2% BSA, and then seeded onto precoated transwells in the same medium alone (control) or in medium supplemented with heparin (1 μg/ml), HRG (20 nM), VEGF (100 ng/ml) or VEGF + heparin. The bottom wells of the transwell contained 600 μl of the same medium as the upper wells but without the cells. After 24 h, the membranes were swabbed with Q-tips and exposed to methanol, followed by treatment with crystal violet prior to counting cell number under a phase-contrast microscope.

RESULTS

Heparin and fibronectin affect VEGF binding to T47D breast cancer cells.

To determine the presence of VEGF receptors on T47D cells, we analyzed the expression of Flk-1/KDR and Flt-4 by Western blotting. Figure 1A indicates that T47D cells express both Flk-1 and Flt-4 receptors as detected by the corresponding specific antibodies. However, we were not able to detect Flt-1 receptors (data not shown).

Heparin has been shown to enhance the binding and to stabilize the complex between VEGF and its receptors in endothelial cells and MDA-MB-231 breast cancer cells (28,30). To test whether heparin and/or fibronectin would affect VEGF binding to T47D cells, we radiolabeled VEGF with [^{125}I]. The ^{125}I-VEGF was then added to the T47D cells and processed for [^{125}I] recovery either after cross-linking (Fig. 1B) or binding (Fig. 1C) to these cells. In the presence of heparin (1 μg/ml), enhanced binding and cross-linking of ^{125}I-VEGF to T47D cells was observed as compared to cells treated with ^{125}I-VEGF alone [824 +/- 69 cpm VEGF vs. 1506 +/- 92 cpm VEGF + heparin (H), $p < 0.01$, Fig. 1B and 1C]. When cells were seeded on fibronectin, an increase in ^{125}I-VEGF binding was observed (1173 +/- 102 cpm, VEGF on FN vs. 824 +/- 69 cpm, VEGF on PL). However, the binding was still lower than that obtained in the presence of heparin. The highest recovery of ^{125}I was observed when heparin was present together with fibronectin, suggesting a synergistic and stronger effect of heparin in this process (1794 +/- 98 cpm). The binding of ^{125}I-VEGF to T47D cells was specific because the addition of a 20-fold excess of nonlabeled VEGF almost completely inhibited formation of the labeled complex (302 +/- 37 cpm, VEGF + VEGF, Fig. 1C).

Effect of ECM on the VEGF-dependent mitogenic response in T47D cells.

Serum-starved T47D cells become synchronized and begin to proliferate uniformly when fed with FBS or growth factors (42). Heregulin is a potent mitogen for breast cancer cells (43,44), and it was used in addition to FBS throughout this study for comparison analysis to VEGF. To study the effect of substratum on mitogenic response, the incorporation of [^{3}H]thymidine into DNA was measured during a 1 h labeling period following the release of cells from quiescence. Untreated T47D cells showed very low [^{3}H]thymidine incorporation (3204 +/- 480 cpm), representing the basal level of DNA synthesis that is marked as 100% (Fig. 2A). After treatment with heregulin, (20 nM), cells grown on plastic slowly increased their incorporation of [^{3}H]thymidine, which peaked between 16 and 20 h (18h = 243 +/- 19%), indicative of the progression through S phase. Cells grown on fibronectin did not significantly affect heregulin-induced DNA synthesis. When cells grown on plastic were treated with VEGF, no significant change in DNA synthesis was observed as compared to that of untreated cells during the course of 24 h (100 +/- 12%; t=0 vs. 117 +/- 22%; t=18h, N.S.). However, cells seeded on fibronectin responded to VEGF by a 2.5-fold increase of [^{3}H]thymidine uptake, over quiescent cells, at the peak of DNA synthesis (242 +/- 11%; t=18 vs. 100 +/- 26%, t=0; $p < 0.01$, Fig. 2A).

At peak incorporation (18 h), the effect of other ECM components, such as matrigel, collagen type I, collagen type IV and poly-L-lysine, was tested and compared to those cells grown on plastic or fibronectin. A significantly low mitogenic response was observed in cells grown on collagen type IV or poly-L-lysine, which was similar to the cells grown on plastic or to untreated (control) cells (Fig. 2B). Cells seeded on matrigel or collagen type I had a comparable increase in VEGF-dependent [^{3}H]thymidine incorporation, which was approximately two-times higher than in the control cells (203 +/- 9 and 206 +/- 13%, respectively). However, fibronectin

caused the most prominent increase in mitogenic response (257 +/- 10%, Fig. 2B), relative to the other matrices. Thus, we have chosen to elucidate the role of fibronectin in VEGF-dependent signaling in breast cancer cells as described below.

The heparin homologue of HSPGs stabilizes the complex between VEGF and its receptors and increases their binding due to several basic residues on both the ligand (45) and receptor (34). Thus, this polyanion was used to test whether it would affect VEGF-dependent mitogenic response in T47D cells. FBS strongly increased [^{3}H]thymidine incorporation at the peak of DNA synthesis (7669 +/- 920 cpm for FBS vs. 3349 +/- 335 cpm for control which is taken as 100%, $p < 0.01$). An additional two-fold increase in DNA synthesis was observed upon FBS treatment of cells that were grown on fibronectin (461 +/- 21%, FBS on FN; vs. 229 +/- 12%, FBS on plastic; $p < 0.01$, Fig. 2C). In the presence of heparin, VEGF was able to increase DNA synthesis 2-3 times that in the control cells (225 +/- 13%, VEGF + H vs. 110 +/- 9%, VEGF alone, vs. 100 +/- 10%, untreated cells), even though cells were seeded on plastic (Fig. 2C). A similar increase in DNA synthesis was observed in cells grown on fibronectin after treatment with VEGF. Furthermore, heparin's presence further increased VEGF-dependent [^{3}H]thymidine incorporation (298 +/- 12%, FN+VEGF+H vs. 246 +/- 11%, FN+VEGF) in T47D cells seeded on fibronectin (Fig. 2C), suggesting a synergistic and stronger effect of heparin. Fibronectin or heparin alone or their combination did not dramatically affect DNA synthesis over the course of 18 h (100 +/- 20%, 95 +/- 11%, and 93 +/- 9%, respectively).

To determine whether the increased mitogenic response was a consequence of more cells initially attaching to fibronectin, cells were labeled with [^{3}H]thymidine for 48 h and passaged onto either fibronectin or plastic. They were allowed to attach for 1 h and the adherent radioactivity was counted. In two experiments, 85 +/- 12% of the plated counts adhered to

plastic compared with 83 +/- 9% of counts on fibronectin. In two additional experiments, nonlabeled, attached cells were harvested and counted directly in a particle counter. Again, 83 +/- 15% of the cells adhered to plastic and 93 +/- 8% to fibronectin. Therefore, the increase in [^{3}H]thymidine incorporation after treatment with VEGF (following a 48-h starvation period) is not due to initial greater adhesion, but rather to an increase of cell mitogenic response in the presence of fibronectin.

Effect of heparin and fibronectin on VEGF-dependent c-fos induction.

Induction of the protooncogene c-*fos* is an early indicator of cell entry into the cell cycle. c-*fos* mRNA levels were reported to be maximal 30-60 min after the stimulation of quiescent breast cancer cells with heregulin (46). Similar amounts of total RNA were loaded in all blots as indicated in the lower panels after probing with an 18S cDNA probe. Cells grown on plastic showed a transient increase in c-*fos* mRNA level in response to VEGF, although it was a much lower response as compared to cells treated with heregulin (Fig. 3A and 3C). In the presence of heparin, a significant increase in VEGF-dependent c-*fos* mRNA level was observed, supporting the results showing a mitogenic response (Fig. 3B). In additional experiments, we further compared c-*fos* mRNA levels 30 min after stimulation with VEGF in cells plated on plastic in the presence of chondroitin sulfate or dextran sulfate or in cells plated on fibronectin in the presence of heparin (Fig. 3B). Dextran sulfate, an unrelated polyanion, increased VEGF-induced c-*fos* transcription similar to heparin, while chondroitin sulfate, a glycosaminoglycan control, did not significantly affect this transcription. Because the c-*fos* mRNA level, in response to dextran sulfate alone, was as high as with VEGF and dextran sulfate together, it suggests that heparin possesses a VEGF-specific mechanism of enhancing the signaling of this growth factor. Cells seeded on fibronectin-coated plates responded to VEGF with an elevated level of c-*fos* mRNA

(Fig. 3B) that was further increased when heparin was present in the medium. This enhancing effect of heparin is similar to that observed when the mitogenic response was tested (Fig. 2C).

We next examined a series of kinase inhibitors to characterize the pathway by which c-*fos* is induced in T47D cells in response to VEGF. Cells were pretreated with genistein, PD98059 or LY294002 before the addition of VEGF and then tested for the level of c-*fos* mRNA (Fig. 3C). While genistein inhibited tyrosine kinases and downstream pathways including MAPK and PI3K, PD98059 inhibited only MEK/MAPK-dependent signaling (47) and LY294002 inhibited only the PI3K/Akt pathway (48). Genistein and PD98059 abolished the VEGF-dependent increase in c-*fos* mRNA level while LY294002 had a modest suppressive effect (Fig. 3C), suggesting the MAPK dependence of c-*fos* induction in these cells.

Heparin and fibronectin affect VEGF-dependent MAPK activation.

Several pathways are known to be involved in c-*fos* induction, with one acting through the Erk family of MAPK (49). Recently, we showed that T47D cells responded to VEGF with a modest MAPK activation which had maximal activity at 15 min (13). Thus, at 15 min, we tested the effects of heparin and fibronectin on VEGF-dependent MAPK activation. Phosphorylation as well as activation of MAPK was analyzed by using phospho-specific antibody and an *in vitro* kinase assay, respectively. Quiescent cells on plastic, fibronectin, or collagen type IV, or cells treated with heparin alone showed very low levels of either MAPK phosphorylation or activity (Fig. 4). When cells were treated with VEGF, a strong increase in the phosphorylation (Fig. 4B) and activity (Fig. 4A) of Erk occurred. However, this activity was still several times lower than that elicited by heregulin. This VEGF-induced increase was sensitive to pretreatment with the MAPK kinase (MEK1/2) inhibitor, PD98059, suggesting a MAPK-dependent process. The response to VEGF was greatly enhanced by the presence of heparin and dextran sulfate, whereas

chondroitin sulfate was without effect (Fig. 4A and 4B). Heparin also slightly increased the level of MAPK phosphorylation in cells seeded on fibronectin in response to VEGF, which otherwise was similar to the cells grown on plastic (Fig. 4B). Consistent with the mitogenic response, MAPK activity was also low on collagen type IV when compared to that on fibronectin (Fig. 4A). The total amount of MAPK protein detected by Western blotting was the same in cells grown on fibronectin or plastic as indicated (lower panels, Fig. 4A and B).

Because VEGF activates the Ras pathway through protein tyrosine kinase receptors (15,50) and subsequently activates MAPK through the dual specificity MEK, extracts of T47D cells rendered quiescent on plastic or on fibronectin were stimulated with VEGF or heregulin and subjected to Western blotting with an anti-phosphotyrosine antibody (data not shown). Four major bands migrating at ~ 60, 80, 125 and 180 kDa were detected. All bands showed basal phosphorylation that was increased after stimulation with VEGF and that was sensitive to genistein. While heparin alone was without effect, heparin or dextran sulfate, together with VEGF, increased the phosphorylation intensity of all bands. Chondroitin sulfate, under the same conditions, was without noticeable effect. Cells grown on fibronectin showed much stronger VEGF-induced phosphorylation of proteins migrating at positions 125 and 180 kDa, when compared to cells seeded on plastic (data not shown). Similar molecular weight protein bands were phosphorylated upon treatment with heregulin. However, the phosphorylation intensity in response to heregulin was much greater than in cells stimulated by VEGF. In addition, heregulin stimulated the tyrosine phosphorylation of a protein at ~70 kDa, which was not seen after cell treatment with VEGF (data not shown).

VEGF-induced PI3K activity is affected by heparin and fibronectin.

Tyrosine phosphorylation of receptors can activate PI3K, which in turn phosphorylates and consequently activates the serine/threonine kinase AKT (51). AKT is activated by its PI3K-dependent recruitment to the plasma membrane that is followed by its phosphorylation at Thr-308 and Ser-473 residues (52). Therefore, measurement of Ser-473 phosphorylated AKT can be an indication of PI3K/AKT pathway activation (52,53). Stimulation of T47D cells by VEGF modestly increased PI3K activity over the basal level, as indicated by the phosphorylation of phospholipids (Fig. 5A) and by AKT phosphorylation (*p-Akt, Fig. 5B). This modest increase in PI3K activity was weaker than in cells stimulated with heregulin (Fig. 5A and 5B). A significant increase in PI3-kinase activation was observed when heparin was present with VEGF in the T47D cells grown on plastic. A slightly higher intensity of VEGF-induced PI3-kinase activity was observed in T47D cells grown on fibronectin (Fig. 5A and 5B). Similar to heparin, dextran sulfate enhanced Akt phosphorylation in the presence of VEGF, while chondroitin sulfate was without effect (Fig. 5B). The increase in VEGF-dependent PI3K activity was completely blocked by pretreatment of cells with the inhibitor LY294002, while PD98059 had no effect. Neither heparin (for both methods), nor dextran sulfate (for the *p-Akt assay, data not shown), alone affected the low basal level of AKT phosphorylation of the quiescent T47D cells. Equal amounts of AKT protein were observed in all samples (Fig. 5B, lower panel).

Effect of heparin and fibronectin on T47D cell migration.

Migration of T47D cells was tested in 24-well plates containing transwell membranes coated with matrigel or matrigel + fibronectin. Approximately 30 to 40 cells migrated onto transwell membranes in the control wells or in wells treated with heparin alone (Fig. 6A and B). When cells were treated with heregulin, a known stimulator of breast cancer cell migration, 104 +/- 11 cells migrated onto the transwell membrane. Upon stimulation with VEGF, cells seeded on

matrigel alone migrated in approximately the same numbers as cells in control wells (Fig. 6A). The migration rate of VEGF-stimulated cells was significantly increased to 78 +/- 4 by the addition of heparin in the moment of cell passage (Fig. 6A). Addition of fibronectin to the matrigel coat further increased the number of migrating cells after treatment with heregulin, VEGF or VEGF plus heparin to 122 +/- 8, 88 +/- 2 and 101 +/- 10, respectively (Fig. 6A). The addition of fibronectin did not affect the migration of nontreated cells or cells treated with heparin alone. To determine the signaling pathway that regulates this migration in T47D cells, cells were pretreated with genistein, PD98059, or with LY294002 prior to treatment with VEGF plus heparin (Fig. 6B). In the presence of heparin and VEGF, 178 +/- 18 cells migrated to the transwell membrane. This migration was decreased by approximately 50% after pretreatment with PD98059. When cells were pretreated with genistein or LY294002, the VEGF-induced migratory response was totally abolished (being 49 +/- 11 and 67 +/- 9, respectively), suggesting both a tyrosine kinase as well as PI3K-dependent process.

DISCUSSION

Our recent study (13), as well as investigations by others (14,54,55), have demonstrated that breast cancer cells secrete VEGF and also express VEGF receptors. However, VEGF alone was not able to induce either a proliferative or migratory response in T47D cells (13). Only cells that were seeded onto fibronectin-coated membranes migrated in significantly higher numbers than cells seeded on matrigel. Given that VEGF and its receptors possess regions rich in basic amino acids that confer binding to the ECM (33,34,45), and that VEGF binds to VEGF receptors in breast cancer cells via its exon 7-encoded ECM-binding region (30), it was reasonable to postulate that VEGF-induced signaling in T47D cells depends on an ECM component. Thus, the effects of heparin and fibronectin on these cells were considered.

VEGF belongs to a FGF group of growth factors that bind heparin. In endothelial cells, VEGF binds with high-affinity to its receptors (28,29). In spite of its high affinity binding, heparin (28,29,34) and HSPGs (27) enhanced this binding and stabilized the complex between VEGF and its receptors. Therefore, it is expected that heparin would support VEGF-induced binding and signaling in T47D cells. Several pieces of information support our hypothesis indicating that an ECM component is required for VEGF-induced mitogenic and migratory response in these cells: i) cells seeded onto plastic showed no significant increase in [^{3}H]thymidine incorporation and no increase in the number of migrating cells after treatment with VEGF on matrigel. Accordingly, T47D cells in response to VEGF on plastic showed very modest increases in overall tyrosine phosphorylation, c-*fos* induction, MAPK activity and PI3K activation as opposed to quiescent cells. The degree of activation was several times lower than that observed in heregulin-treated cells; ii) heparin presence and/or attachment to fibronectin-coated plates was sufficient to establish the mitogenic response and increase migration of these

breast cancer cells in response to VEGF similar to the response elicited by heregulin, a potent breast cancer cell mitogen; iii) signaling pathways that lead to proliferation such as MAPK and c-*fos* or that stimulate cell migration, such as PI3K, were significantly increased in response to VEGF when cells were exposed to heparin and/or seeded onto fibronectin-coated dishes; and iv) as suggested by Soker et al. (30), heparin/HSPGs enhance binding of VEGF to its receptors. Thus, in order to stimulate signaling in T47D cells, the binding of VEGF to its receptors needs to be stabilized by the presence of ECM components such as heparin and fibronectin.

Although the binding of both VEGF and its receptors to heparin has been suggested by several authors (28,29,34), their binding to fibronectin and its stabilizing role in the VEGF-VEGF-receptor complex have not been reported to date. Fibronectin has been shown to augment cell migration (56). Fibronectin contains several binding domains including those for fibrin, collagen, heparin and cell-surface receptors (57). Its two heparin-binding domains, one of low affinity and the other of high affinity, are located at its N- and C-terminus, respectively (57). The binding of a heparin-binding domain of fibronectin to cell surface HSPGs promotes focal adhesion formation by activating PKC (58). This result was duplicated in our system, as cells seeded on fibronectin showed the increased tyrosine phosphorylation of a protein migrating at ~120 kDa. Although the nature of that band was not further investigated, its molecular weight and the fact that it is more phosphorylated in cells grown on fibronectin suggest that this band could be FAK. Activated PKC and FAK could lead to MAPK activation, which induces c-*fos* transcription such as observed in our present work. Alternatively, fibronectin alone or together with HSPGs could stabilize the complex between VEGF and its receptors. The effect of fibronectin also could be attributed to the specific peptide PHSRN contained in its molecule, that was recently reported to accelerate wound healing by increasing cell invasiveness (59). Further

study will be required to fully address the effects of fibronectin on the VEGF receptor-growth factor complex.

The strong suppressive effect of poly-L-lysine on DNA synthesis suggests the involvement of integrins, which may mediate signaling from matrices containing matrigel or collagen type I. Integrins might also play a role in inducing the inhibitory effect of collagen type IV. Collagen type IV was shown to inhibit proliferation of melanoma cells (60) and breast cancer cells (61) by up to 67%. This inhibition was attributed to the Serine-N-Serine sequence of the α3(IV) collagen chain. Integrin $\alpha v\beta 3$ and integrin-associated protein CD47 were also responsible for this effect (62). Although collagen type IV peptides were added to the growth medium (62), it is conceivable that in our experiments (where cells were seeded onto a collagen type IV coat) cells were also exposed to the α3(IV) chain, which exerted its anti-proliferative effect.

Signal transduction by tyrosine kinase receptors, such as VEGF, depends upon the binding of SH2-SH3 domain-containing proteins to tyrosine phosphorylation sites on the receptor (10). Subsequent MAPK activation converges from several pathways, one emanating from the activation of PLC-γ through PKC and, another, through the Ras/MEK-dependent cascade (17). Decreased MAPK activity, such as that observed in VEGF-treated T47D cells grown on plastic, is consistent with low mitogenic response and weak c-*fos* induction. Strong and sustained activity of MAPK is required for its translocation to the nucleus where the enzyme activates transcription factors (63). Likewise, as indicated in the present study, plating of T47D cells onto fibronectin, or onto plastic in the presence of heparin, significantly increased all the signaling components tested in response to VEGF, providing the additional requirements for cells to complete passage through the S-phase. The phosphorylated VEGF receptor can also

recruit the p85 subunit of PI3K (17,64), thereby activating the PI3K/AKT pathway. Activated PI3K/AKT may regulate cell survival (52), proliferation (65,66), and migration (67). We have observed migration of T47D cells in response to VEGF only when heparin and/or fibronectin was present on the transwell membrane, suggesting that both the growth factor and the ECM components are important in potentiating the migration of tumor cells. In endothelial cells, the PI3K pathway was shown to be crucial in cell survival (68) and to play an important role in the regulation of cell migration (4,67). Similarly, in our system, we found that PI3K is involved in the regulation of cell survival (13) as well as in the process of cell migration, because LY294002, a PI3K specific inhibitor, strongly inhibited the VEGF-dependent migratory response while PD98059, a specific MEK inhibitor, had a moderate effect. Our results are consistent with those of Adam et al, who reported that MCF-7 cells, another breast cancer cell line, migrated in response to heregulin through a PI3K-mediated process (69).

Induction of c-*fos* by growth factors operates through initial tyrosine phosphorylation of activated receptors, leading to subsequent MAPK activation. This induction is sensitive to inhibition of either tyrosine phosphorylation or MEK. Activation of MAPK leads to phosphorylation of the transcription factor Elk, which drives transcription of c-*fos* through serum response elements in the c-*fos* promoter (70). PI3K was also shown to contribute to the cell cycle by inducing the AKT-dependent phosphorylation of glycogen synthase kinase 3 (71) and by activating protein-serine/threonine $p70^{S6}$ kinase (65). However, our results strongly suggest that the PI3K-dependent pathway does not play a significant role in VEGF-induced c-*fos* transcription in T47D cells. First, the c-*fos* mRNA level was not markedly reduced by pretreatment with LY294002, whose specificity to PI3K has been demonstrated (48). Additionally, the VEGF-dependent increase in the level of c-*fos* mRNA was abolished after cell

pretreatment with PD98059. Finally, corroborating our results, it was recently reported in the HUVEC system that the PI3K and MAPK pathways do converge (66). However, PI3K was shown to be upstream of MAPK and to only affect induction of c-*fos* through the regulation of MAPK activity. The significant increase in c-*fos* transcription in response to VEGF could be attributed to the specific effect of heparin because dextran sulfate alone, an unrelated polyanion, caused a strong increase in c-*fos* induction. Chondroitin sulfate, a control for GAG chains, was without effect either on c-*fos* induction or MAPK or PI3K activation. The induction of c-*fos* transcription by dextran sulfate alone supports the notion that part of heparin's effect is through electrostatic stabilization of the binding complex. For example, the binding affinity of FGFs to heparin, which is a highly sulfated GAG, is greater than that to heparan-sulfate, a GAG that is less sulfated (45). However, the high isoelectric point, by itself, is insufficient to explain the binding, because PDGF, with an isoelectric point similar to b-FGF, does not localize to anionic heparan sulfate sites on the basement membrane of the eye, whereas a-FGF and b-FGF do bind to the same sites (72).

Extracellular matrix-induced oligomerization appears to be an important mechanism in regulating the biological activities of growth factors (73). Since breast cancer cells express Flt-1 and Flk-1/KDR receptors (13,55,74) and secrete VEGF (13,54), this phenomenon may emphasize an autocrine mechanism by which VEGF increases the tumorigenicity of breast cancer cells. In the early stages of breast cancer development, there is an initial accumulation of ECM components in both the interstitial stroma (75) and in the basement membrane (76). Accumulated ECM, such as fibronectin and HSPGs, may increase the responsiveness of breast cancer cells to VEGF by maintaining the stability of the complex between the growth factor and its receptor resulting in receptor activation. This could increase tumorigenicity through the

stimulation of cell proliferation, survival and invasiveness. However, this initial phase does not seem to last very long due to a general decrease in ECM components and in the level of sulfation of heparan sulfate, which has been associated with the metastatic phenotype of cancer cells. This final stage of transformation can be ascribed to the upregulation of ECM-degrading matrix metalloproteinases (77).

In this report, we demonstrated that VEGF requires the presence of ECM components for the induction of mitogenic and migratory responses in T47D cells. As compared to T47D cells grown on plastic, cells seeded on fibronectin or cells grown on plastic in the presence of heparin had significantly higher [^{3}H]thymidine incorporation and c-*fos* induction as well as a higher number of cells migrating to the transwell membranes. Likewise, intracellular signaling that supports mitogenic and migratory responses, such as general tyrosine phosphorylation, MAPK activity, and PI3K activity, was much greater in cells seeded on fibronectin or seeded on plastic in the presence of heparin, as compared to cells grown on plastic. Although heparin has been shown to enhance the binding of VEGF to its receptors in both breast cancer (30) and endothelial cells (28), this is the first report associating ECM enhancement of VEGF binding to the induction of mitogenic and migratory responses in breast cancer cells.

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FIGURE LEGENDS

Figure 1. Heparin and fibronectin enhance binding of VEGF to T47D cells.

A) T47D cells were seeded onto 100 mm Petri dishes and total cell lysates were subjected to immunoblotting by either anti-Flk-1, anti-Flt-4, or control antibodies. As a control for loading, the same blots were stripped and probed by monoclonal anti-actin antibodies (Actin).

T47D cells were grown to confluency either in 6-cm plates (B) or in 24-well plates (C), washed with PBS and treated with ^{125}I-VEGF for 4 h in an ice-cold environment. B) Cells seeded on plastic (lanes 1 and 2) or on fibronectin (lanes 3 and 4) were treated with ^{125}I-VEGF alone (lanes 1 and 3) or treated with ^{125}I-VEGF in the presence of heparin (lanes 2 and 4, 1 μg/ml). After cross-linking the bound ^{125}I-VEGF, cells were scraped as described in Experimental Procedures and subjected to SDS-PAGE followed by autoradiography. Molecular weight markers are indicated to the right. C) Cells seeded on plastic (PL) or fibronectin (FN) were treated with ^{125}I-VEGF alone, with ^{125}I-VEGF in the presence of heparin (VEGF +H), or in the presence of 20-times excess of unlabeled VEGF (VEGF + VEGF). Cells were then washed with ice-cold PBS and processed for recovery of bound ^{125}I radioactivity as described in Experimental Procedures. "0" indicates background radioactivity recovered from non-treated cells. Values in graph represent the mean +/- S.D. of two experiments. Autoradiographs are also representative of two separate experiments.

Figure 2. Fibronectin and heparin affect VEGF-dependent DNA synthesis in T47D cells.

T47D cells seeded onto plastic (PL) or fibronectin (FN)-coated 24-well plates were starved and treated either with VEGF, heregulin (HRG) or FBS (10%) in the presence or absence of heparin (H). Cells were then processed for determination of incorporated radioactivity as described in Methods. A) Cells seeded on PL (○ , □) or on FN (● , ■) were treated with VEGF (○ , ●) or

HRG (□,■) for the indicated times. Values are expressed as the means (%) +/- SD of control, non-treated cells (taken as 100%), from quadruplicate wells of 3 separate experiments. B) Cells were seeded on plastic (PL), fibronectin (FN), Matrigel (MTR), collagen types I and IV (COL-1, COL-4) and poly-L-lysine (POL-L) treated with VEGF (100 ng/ml) and processed for determination of the incorporated radioactivity at peak value (18 h, see Fig. 2A). C) Cells were seeded on PL or FN-coated wells, and incorporated radioactivity at peak value (18 h, see Fig. 2A) after treatment with VEGF, FBS, heparin (H), or VEGF +H was compared to the non-treated cells (control, taken as 100%). Values are the means (%) +/- SD of control from quadruplicate wells of two separate experiments.

Figure 3. Heparin and fibronectin enhance VEGF-dependent c-*fos* induction in T47D cells. Quiescent T47D cells seeded on plastic or FN-coated plates were stimulated at time 0 with VEGF or with heregulin (HRG). A) Cells were treated with VEGF at the indicated time points or with HRG as a control and total RNA was collected and subjected to Northern blotting. B) Total RNA was collected immediately or after 30 min of treatment with VEGF in the presence or absence of heparin, dextran sulfate (DS), or chondroitin sulfate (CS), or C) kinase inhibitors such as genistein (GEN, 50 μM), PD98059 (PD, 50 μM) and LY294002 (LY, 10 μM). Blots shown in A, B and C were probed with cDNAs to c-*fos* (upper panels) and to 18S rRNA (lower panels). Results are representative of three independent experiments.

Figure 4. Effect of heparin and fibronectin on VEGF-induced MAPK phosphorylation and activation in T47D cells.

T47D cells were seeded onto PL, collagen type IV (COL) or FN-coated plates and starved as indicated in Fig. 2. A) Cell treatment was followed by immunoprecipitation with anti-Erk-2 antibody. Immunoprecipitates were then used in an *in vitro* kinase assay with myelin basic

protein (MBP) as a substrate. Control indicates kinase activity of the sample containing lysate from cells treated with VEGF mixed with protein-G agarose without anti-Erk-2 antibody. The lower panel represents immunoblots of immunoprecipitates probed with anti-Erk-2 antibody. B) Blots of total cell lysates were probed with anti-phospho-Erk-2 antibodies (*p-Erk, upper panel), and then were stripped and reprobed with anti-Erk-2 antibody as a control for total Erk protein (lower panel). Blots are representative of two independent experiments.

Figure 5. Effect of heparin and fibronectin on VEGF-dependent PI3K activity.

Cells were seeded on PL or FN as described in Experimental Procedures. After 15-min treatment with VEGF, VEGF in the presence of heparin (VEGF + H), chondroitin sulfate (CS), or dextran sulfate (DS), or after treatment with heregulin (HRG), or with heparin alone, total cell lysates were subjected to either *in vitro* PI3K assay (A) or to immunoblotting (B). Untreated cells (control) or cells pretreated with either PD98059 (50 μM, PD) or LY294002 (10 μM, LY) were subjected to treatment with VEGF. A) Immunoprecipitates obtained with anti-phosphotyrosine antibodies were used to phosphorylate lipids in the presence of $\gamma[^{32}P]$ATP in an *in vitro* PI3-kinase assay. The phosphorylated lipids were then extracted from the reaction mixture by $CHCl_3$:CH_3OH [1:1], spotted onto TLC plates, separated in developing solution containing $CHCl_3$:CH_3OH:NH_4OH:H_2O [60:47:11,3:2] followed by autoradiography. "Blank" represents the reaction mixture of HRG-treated cells treated that was precipitated with Protein-G sepharose without anti-phosphotyrosine antibody. The autoradiograph is representative of two separate experiments. B) Immunoblots were probed with anti-p-Ser^{473} AKT antibody (*p-AKT, upper panel) or with anti-AKT antibodies (lower panel). The blots are representative of two separate experiments.

Figure 6. Heparin and fibronectin affect the VEGF-induced migratory response of T47D cells.

Cells were resuspended in DMEM containing 0.2% BSA and seeded onto membranes precoated either with matrigel (MTR) or with a mix of matrigel and fibronectin (MTR + FN), as indicated in Experimental Procedures. A) In the moment of passage, the cell suspension contained only BSA (control), heparin (H), HRG, VEGF, or VEGF + H. B) Cells were treated with VEGF plus kinase inhibitors such as genistein (GEN), PD98059 (PD), or LY294002 (LY) for half an hour prior to passage in the presence of heparin (H) and compared to those treated only with heparin (H) or with VEGF in the presence of heparin (VEGF + H). Values represent the means +/- SD of triplicate wells from three independent experiments.

Fig. 1

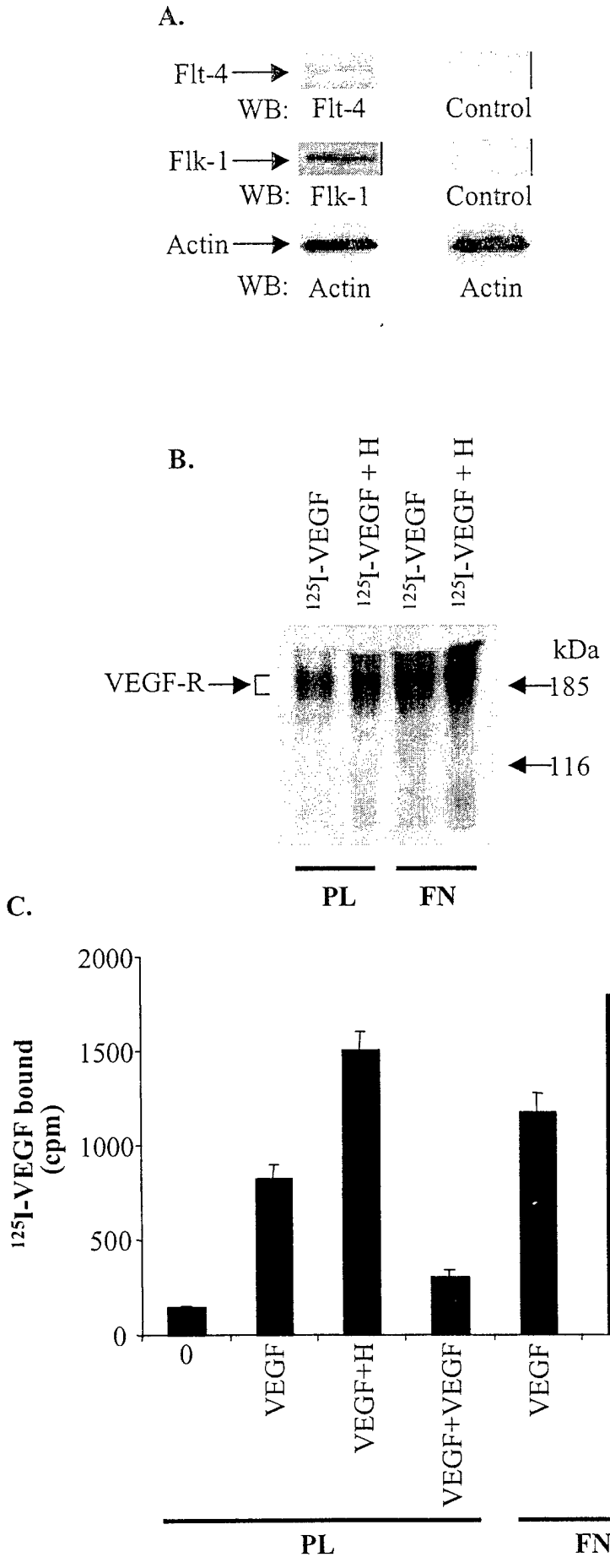
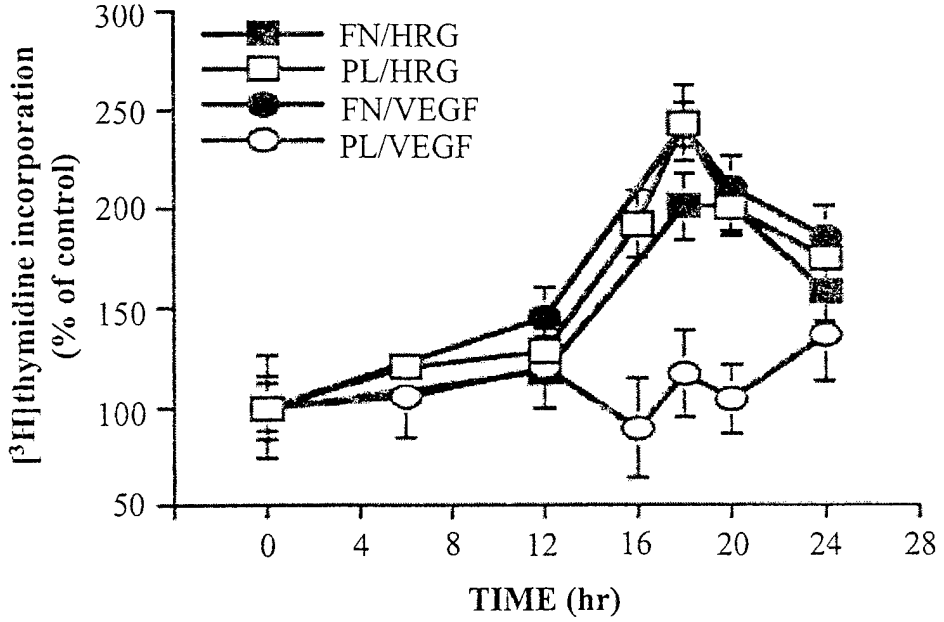
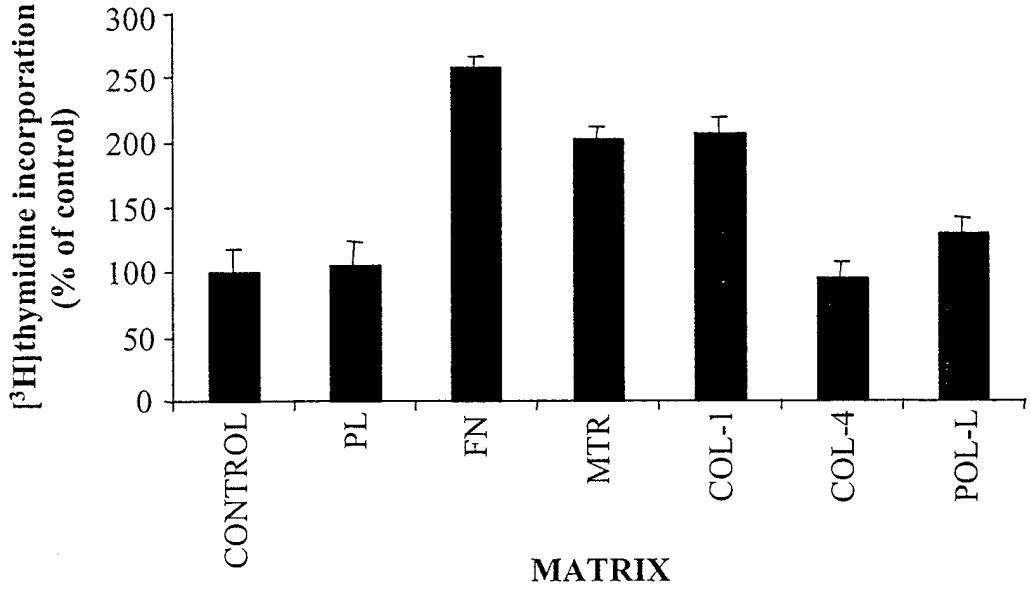

A.
Flt-4
WB: Flt-4
Control
Flk-1
WB: Flk-1
Control
Actin
WB: Actin
Actin
B.
125I-VEGF
125I-VEGF + H
125I-VEGF
125I-VEGF + H
kDa
VEGF-R
185
116
PL
FN
C.
125I-VEGF bound (cpm)
2000
1500
1000
500
0
0
VEGF
VEGF+H
VEGF+VEGF
VEGF
VEGF+H
PL
FN

Fig. 2

A.


FN/HRG
PL/HRG
FN/VEGF
PL/VEGF
[3H]thymidine incorporation (% of control)
300
250
200
150
100
50
0
4
8
12
16
20
24
28
TIME (hr)

B.


[3H]thymidine incorporation (% of control)
300
250
200
150
100
50
0
CONTROL
PL
FN
MTR
COL-1
COL-4
POL-L
MATRIX

C.

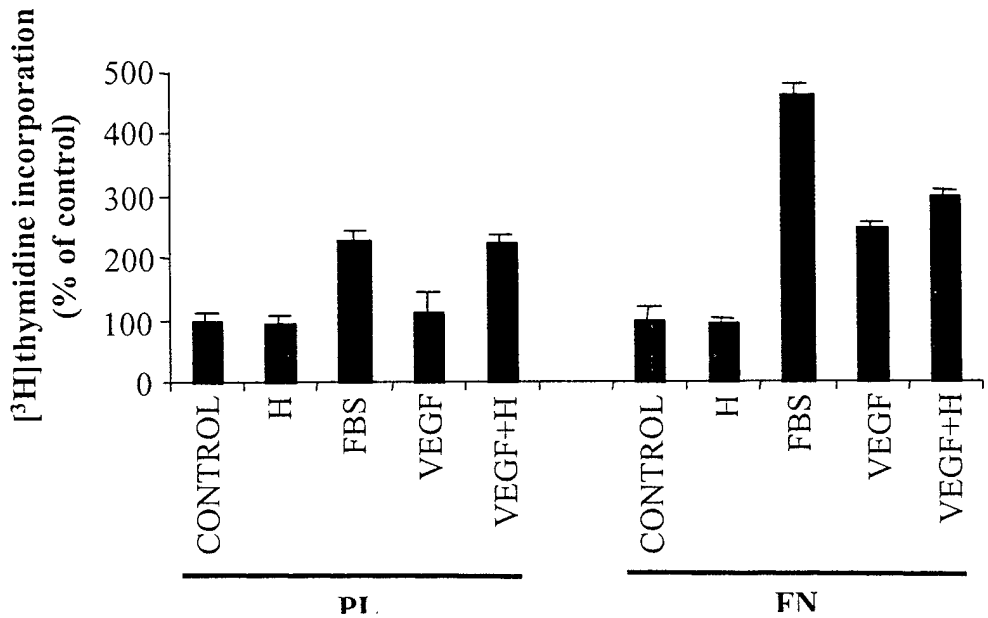
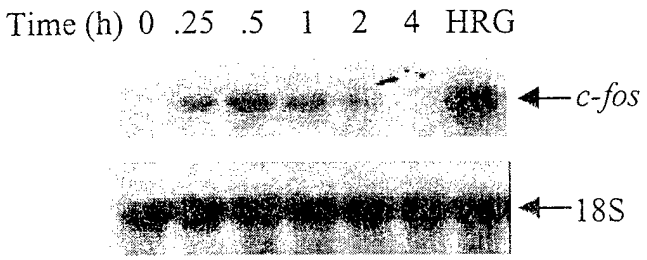
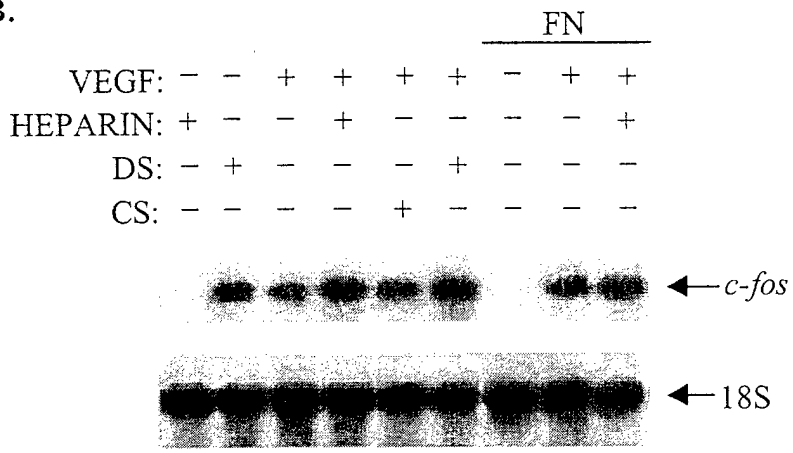

[3H]thymidine incorporation (% of control)
500
400
300
200
100
0
CONTROL
H
FBS
VEGF
VEGF+H
CONTROL
H
FBS
VEGF
VEGF+H
PL
FN

Fig. 3

A.


Time (h) 0 .25 .5 1 2 4 HRG
c-fos
18S

B.


FN
VEGF: − − + + + + − + +
HEPARIN: + − − + − − − − +
DS: − + − − − + − − −
CS: − − − − + − − − −
c-fos
18S

C.

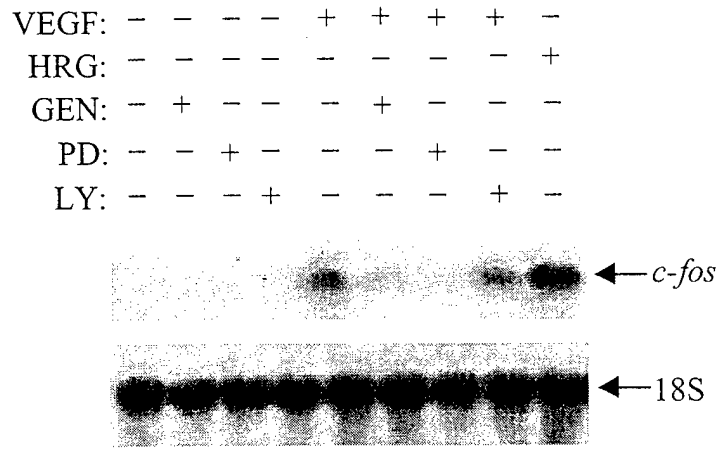

VEGF: − − − − + + + + −
HRG: − − − − − − − − +
GEN: − + − − − + − − −
PD: − − + − − − + − −
LY: − − − + − − − + −
c-fos
18S

Fig. 4

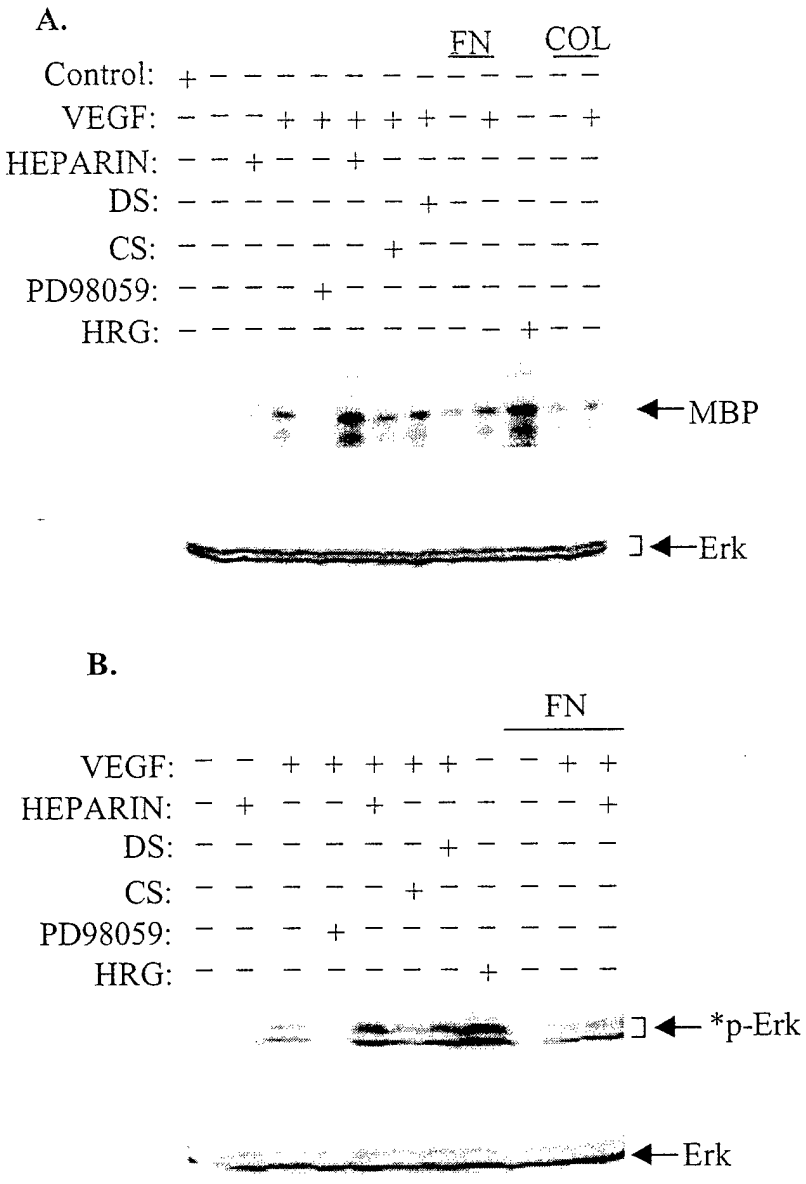
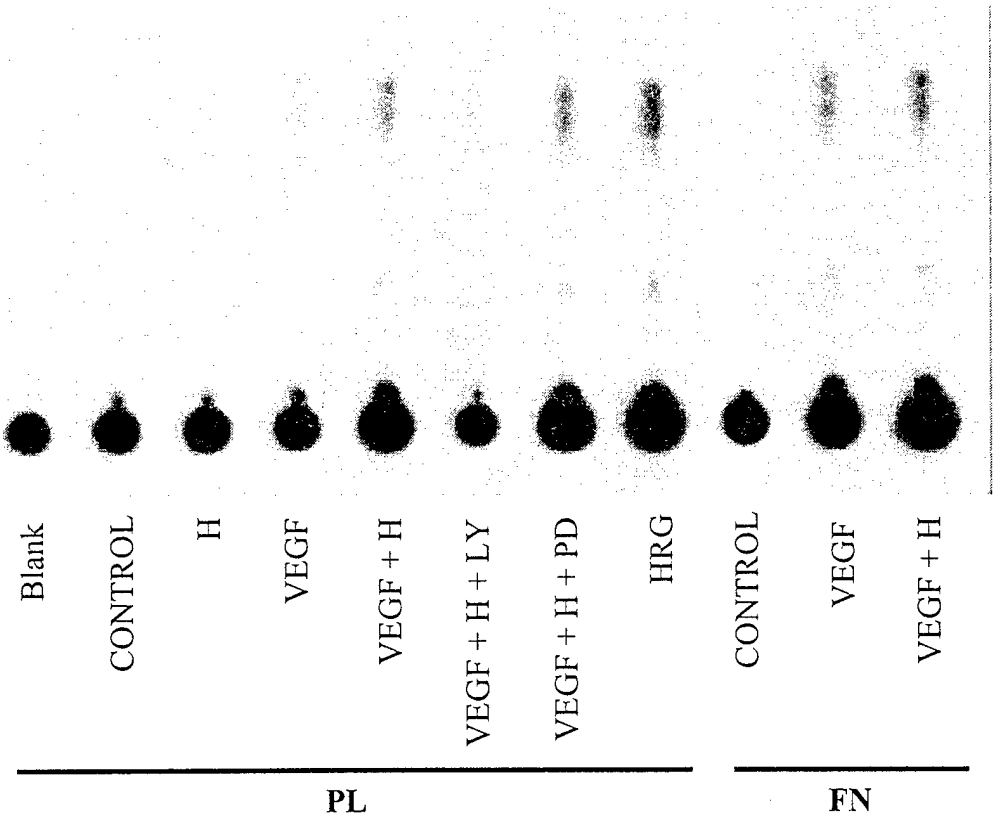

A.
FN
COL
Control: + − − − − − − − − − − −
VEGF: − − − + + + + + − + − − +
HEPARIN: − − + − − + − − − − − − −
DS: − − − − − − − + − − − − −
CS: − − − − − − + − − − − − −
PD98059: − − − − + − − − − − − − −
HRG: − − − − − − − − − + − −
MBP
Erk
B.
FN
VEGF: − − + + + + + − − + +
HEPARIN: − + − − + − − − − − +
DS: − − − − − − + − − − −
CS: − − − − − + − − − − −
PD98059: − − − + − − − − − − −
HRG: − − − − − − − + − − −
*p-Erk
Erk

Fig. 5

A.


Blank
CONTROL
H
VEGF
VEGF + H
VEGF + H + LY
VEGF + H + PD
HRG
CONTROL
VEGF
VEGF + H
PL
FN

B.

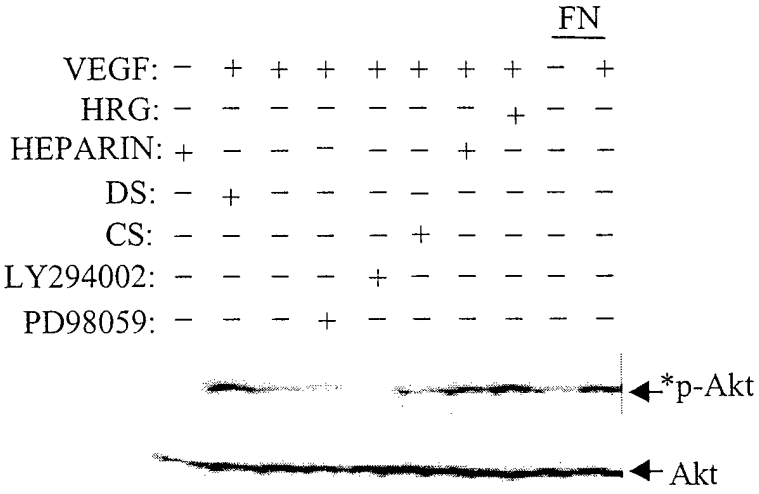
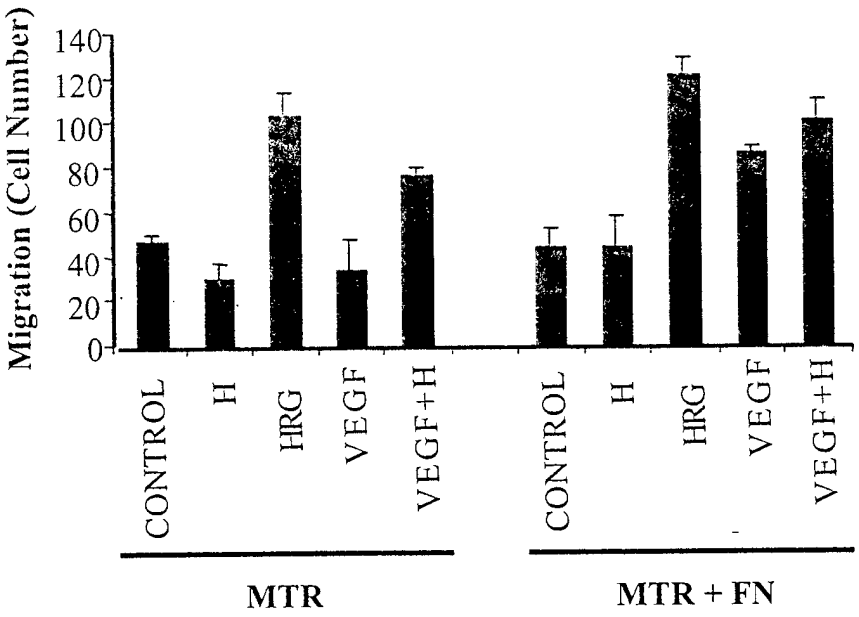
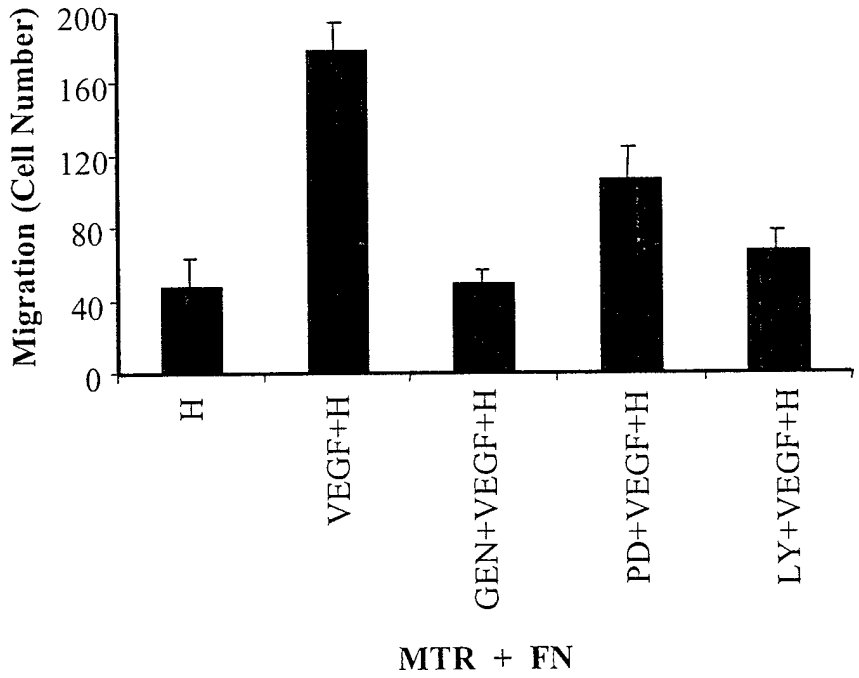

FN
VEGF: − + + + + + + + − +
HRG: − − − − − − − + − −
HEPARIN: + − − − − − + − − −
DS: − + − − − − − − − −
CS: − − − − − + − − − −
LY294002: − − − − + − − − − −
PD98059: − − − + − − − − − −
*p-Akt
Akt

Fig. 6

A.


Migration (Cell Number)
140
120
100
80
60
40
20
0
CONTROL
H
HRG
VEGF
VEGF+H
CONTROL
H
HRG
VEGF
VEGF+H
MTR
MTR + FN

B.


Migration (Cell Number)
200
160
120
80
40
0
H
VEGF+H
GEN+VEGF+H
PD+VEGF+H
LY+VEGF+H
MTR + FN